昭和32年7月9日第三種郵便物認可
第48巻第5号昭和35年11月25日発行
(毎月1回25日発行)
The Journal of Biochemistry, published monthly by
the Japanese Biochemical Society, Tokyo, Japan.
Vol. 48, No. 5, November 25, 1960

Vol. 48, No. 5 **November, 1960**

THE JOURNAL OF BIOCHEMISTRY

EDITED FOR THE JAPANESE BIOCHEMICAL SOCIETY

EDITORIAL BOARD

SHIRO AKABORI
TOSHIO ANDO
SABURO FUNAHASHI
KOOZOO KAZIRO
BUNJI MARUO
DEN'ICHI MIZUNO
NORIO SHIMAZONO
HIROSHI TAMIYA
ITARU WATANABE
SHICHIRO AKIYA
FUJIO EGAMI
OSAMU HAYAISHI
KATASHI MAKINO
TOMOO MIWA
ZIRO NIKUNI
YUSUKE SUMIKI
TYUNOSIN UKITA
HARUHISA YOSHIKAWA

PUBLISHED MONTHLY
BY
THE JAPANESE BIOCHEMICAL SOCIETY
Tokyo University, Tokyo

AGENCIES

Charles E. Tuttle
Rutland, Vt.
U.S.A.

Maruzen Co., Ltd.
Tokyo
Japan

Santo Vanasio
Köln
Germany

PRINTED IN JAPAN

THE JOURNAL OF BIOCHEMISTRY was founded in 1922 by Prof. emeritus Dr. S. Kakiuchi and had been edited by him to volume 36 of the year of 1944. After the discontinuation during the War, this JOURNAL has been restarted by The Japanese Biochemical Society, following the serial number from volume 37 of the year of 1950.

This JOURNAL is collecting the original investigations on the subject in this country. It is published monthly, one volume per 6 months.

Note to Contributors

All manuscripts should be carefully typewritten, triple-spaced, in English, French or German, and addressed to *The Editor, The Journal of Biochemistry, The Japanese Biochemical Society, c/o Department of Biochemistry, Faculty of Medicine, Tokyo University, Bunkyo-ku, Tokyo, Japan.*

Authors receive 50 reprints of their papers as a rule at cost; additional copies can be prepared by the order on cost accounts. All orders for reprints should be markedly noted on the article paper or in a sheet of paper attached to it. On submitting the paper, it is preferable to attach the non-traced charts, besides, original drawings, for circulation to Editors.

On request from authors to contribute to JOURNAL, the *Directions for preparation of manuscripts*, including styles of abbreviations of symbols, terms, units, and periodicals will be sent immediately.

Subscription

Subscription Price. ¥ 2,700 (U.S. $ 7.50) per volume, postage inclusive. Back issues are available for sale, from volume 1 to volume 46 at ¥ 3,600 (U.S. $ 10.00) per volume, postage inclusive.

Remittances should be made preferably by check or draft payable to *The Japanese Biochemical Society, c/o Department of Biochemistry, Faculty of Medicine, Tokyo University, Bunkyo-ku, Tokyo, Japan.*

Foreign subscription is treated by the agencies of *Charles E. Tuttle, Co.*, Rutland, Vermont, U.S.A., *Santo Vanasio*, Köln, Germany, and *Maruzen Co., Ltd.*, Chuo-ku, Tokyo, Japan.

The Journal of Biochemistry, Vol. 48, No. 5, 1960

DETERMINATION OF COENZYME A BY AZOTOMETRY*, **

By TOMOMI SAKURADA

(*From the Department of Biochemistry, School of Medicine, Keio University, Tokyo*)

(Received for publication, January 20, 1960)

Enzymic reactions involving coenzyme A*** to give active acyl groups are especially significant in intermediating group transfer in metabolism. Acetylation of an amino group *in vivo* has been shown to be dependent on CoA function, and Kaplan and Lipmann (*1*) used the enzymic acetylation of sulfanilamide to determine CoA. Their method has a greatly contributed to advance the studies of CoA. It has been proved that acetylation of INAH is also due to enzymic action involving CoA (*2*). The reported characteristics of Iwasaki's azotometry towards INAH and its derivatives (*3*) and the successful modification of the azotometry for application to a smaller-scale analysis (*4*) are the basis of the method for determination of CoA proposed in this paper. The method may provide additional means to further studies of CoA functions.

MATERIALS AND APPARATUS

Azotometry—Apparatus: The azotometer used was with one eudiometer of 50-λ, graduated to 0.5 λ, or with two eudiometers, one of 12-λ, graduated to 0.1 λ, and the other of 2-λ, graduated to 0.025 λ.

Kipp's apparatus for carbon dioxide generation, wash bottle and accessory equipment, and pressure device to pass CO_2 against the higher resistance in the azotometer were all modified.

Reagents: Saturated saline solution

10 N sodium hydroxide

5 per cent potassium ferricyanide solution in saturated sodium nitrate.

5 per cent potassium dichromate solution dissolved in saturated sodium nitrate, with 1/99 volume of 10 N sulfuric acid added to bring the final concentration of the acid to 0.1 N.

INAH: Commercial product was used *per se*. Analysis (Micro Dumas): Calcd.

---

* This paper written in Japanese was published in *J. Japan. Biochem. Soc.*, **31**, 389 (1959).

** A part of expenses for this work was defrayed by the Grant-in-Aid for the Development Scientific Research from the Ministry of Education which is gratefully acknowledged.

*** The abbreviations used are: CoA, coenzyme A; INAH, isonicotinic acid hydrazide; Acetyl-INAH, acetyl isonicotinic acid hydrazide; TCA, trichloroacetic acid.

for $C_6H_7ON_3$: N 30.15. Found: N 29.74.

Acetyl-INAH: INAH was acetylated with acetic anhydride and recrystallized from ethanol (*5*). Analysis (Micro Dumas): Calcd. for $C_8H_9O_2N_3$: N 23.45. found: N 23.67.

METHOD OF DETERMINATION

Azotometry of INAH or Acetyl-INAH—Figures in parentheses indicate the value to be used in case of an azotometer with two eudiometers.

1) Ferricyanide Azotometry: One (0.5) ml. of the sample in a small beaker is taken into the reaction tube of azotometer by suction, followed by 7 (1.5) ml. of saturated saline solution to rinse the beaker, and CO_2 is passed through the solution for 5 (10) minutes (during this time, CO_2 is also bubbled into the potassium ferricyanide solution). Into this azotometer, 0.5 (0.2) ml. of this ferricyanide solution are added, the contents are mixed thoroughly, and 1.0 (0.3) ml. of 10 *N* NaOH is added from a small beaker. The whole is shaken for 2 minutes. Fresh 10 *N* NaOH must be used each time. Azotometry by the usual procedure is performed, and the amount of INAH or Acetyl-INAH is calculated from the volume of nitrogen gas evolved.

2) Dichromate Azotometry: One (0.5) ml. of the sample is placed in the azotometer with 7 (1.5) ml. of saturated saline solution as before and CO_2 is passed through this solution for 5 (10) minutes. To this is added 1.0 (0.3) ml. of potassium dichromate solution, preliminarily bubbled with CO_2 to remove dissolved air and the whole is shaken for 2 minutes. When the internal pressure becomes positive suction is applied to make the internal pressure negative. One (0.3) ml. of 10 *N* NaOH is added, the mixture is shaken for 1 minute, and then processed by the usual method of azotometry to determine the volume of nitrogen gas generated from INAH.

3) Azotometry of Acetyl-INAH: This is the combination of the above two methods. When it is desired to determine only Acetyl-INAH from a mixture of INAH and Acetyl-INAH, the procedure can be more simplified and the desired value can be obtained from one course of azotometric procedure. The resultant value so obtained is the same as that obtained from the difference between the values of the ferricyanide and of dichromate methods. In this case, 1.0 (0.5) ml. of the sample solution and 1 (0.3) ml. of the dichromate solution are placed in the azotometer with 6 (1.2) ml. of the rinse solution, CO_2 is passed through the solution for 5 (10) minutes, and this is submitted to the ferricyanide azotometry. In such a manner, only free INAH is quantitatively oxidized by dichromate during the passage of CO_2 and the Acetyl-INAH which remains unchanged, is subsequently determined by ferricyanide azotometry. By this procedure it is possible to attain a selective determination for Acetyl-INAH.

The results of determinations of INAH and Acetyl-INAH by the foregoing methods of ferricyanide or dichromate azotometry with samples in the range of 1-10 μg. of nitrogen in the hydrazide portion, is shown in Fig. 1.

It is seen from these results that, while all the nitrogen in the hydrazide portion in INAH is liberated as nitrogen gas by both the ferricyanide and dichromate methods, all the hydrazide-nitrogen in Acetyl-INAH is liberated only by the ferricyanide method and not by the dichromate method. Consequently, it becomes possible to effect separate determinations of each component from a mixture of INAH and Acetyl-INAH by azotometry. The use of this specificity permits a reliable following of the formation of Acetyl-INAH during the enzymic acetylation of INAH which is the progress of the enzymic reaction. Since the rate of this enzymic reaction is related to the amount of CoA, a means provided for its measurement by azotometry.

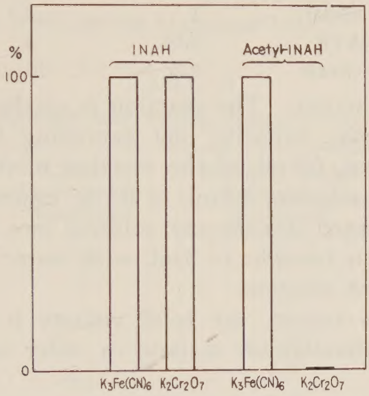


FIG. 1. Specificity of INAH and Acetyl-INAH to oxidation.

Ordinate shows the percentage of N_2 gas evolved.

Preliminary Treatment in the Determination of Coenzyme A—1) Apoenzyme: In accordance with the method of Kaplan and Lipmann (*1*), an acetonedried powder of pigeon liver was prepared and extracted with 10 volumes of 0.02 *M* $NaHCO_3$ solution. The insoluble portion was discarded after centrifugation, and the supernatant solution was frozen with a freezing mixture and kept over-night in the freezing portion of a refrigerator. After thawing and standing for 4 hours at 25° in order to effect aging, the solution was centrifuged to remove insoluble matter and stored in an ice chamber.

2) Standard CoA: Prepared as a dried powder from albino rat liver (*1*). This crude CoA preparation contained 1.07 unit per mg. of dry matter.

3) Reaction mixture: It is more convenient to keep prepared a reaction mixture of most of the reagents. The composition of the reaction mixture is listed below and it contains INAH as a substrate for acetylation. The Na ATP used in this work was a product of Nutritional Biochemicals Corporation or prepared from rabbit muscles according to the method of

Needham (*6*), the latter being of 60 per cent purity by chemical examination.

Substance	*M*	ml.
INAH	0.06	10
Na acetate	1	2.5
Na ATP	0.05	8
Na citrate	0.2	10

4) Acetylation reaction: The reaction is carried out as follows: In a test tube of 16×100 mm. capacity, not exceeding 0.75 ml. of the sample solution containing CoA, 0.9 ml. of the reaction mixture, 0.24 ml. of freshly prepared *M* $NaHCO_3$ solution, 0.3 ml. of 0.1 *M* cysteine hydrochloride solution, and 0.75 ml. of aged apoenzyme solution are placed in that order, and the total volume is brought to 3 ml. with water. For the blank, water is used in place of CoA solution.

In such a reaction system, the total volume is just three times that used in the case of sulfanilamide method in order to comply with azotometric procedure.

The test tube is glass stoppered, the contents are mixed thoroughly, and incubated at 37° for 2 hours.

The reaction is then stopped by the addition of 0.9 ml. of 16 per cent TCA, the solution is centrifuged, and the supernatant (volume, 3.3–3.6 ml.) is separated. Addition of its 77/1000 volume of 4 *N* NaOH solution will make the solution neutral or weakly alkaline. This neutralization brings 1 ml. of the acetylation reaction mixture to a volume of 1.4 ml.

Azotometry—One (0.5) ml. of the neutralized solution thus obtained by preliminary treatment is placed in a small beaker and submitted to the ferricyanide or dichromate azotometry. The difference in the values of the two is the amount of Acetyl-INAH formed. The amount of Acetyl-INAH can also be obtained directly by the combined method. For the sake of convenience the amount of INAH or Acetyl-INAH will be expressed in terms of λ equivalent to the nitrogen of the hydrazide moiety.

DISCUSSION

The acetylation reaction was carried out as stated above and the effect of varying the amout of standard CoA or apoenzyme on the acetylation was examined.

a) *Effect of Varying Amounts of CoA on Acetylation*—Results are shown in Table I and Fig. 2.

These results indicate that acetylation of INAH proceeds in approximate proportion to the amount of CoA at medium concentrations of CoA. The maximum amount of acetylation is 7.4 λ in N_2 at which the rate of acetylation is 79 per cent of the INAH added. Therefore, the amount of CoA required to effect 50 per cent acetylation of the maximum rate may be taken as the standard of unit as in the case of Kaplan and Lipmann (*1*). The amount of CoA preparation which acetylates INAH to 3.7 λ is

2.8 mg. from the curve in Fig. 2. As stated above, the present method requires a reaction mixture of three times greater in volume than in the sulfanilamide method, so 2.8/3 mg. of the preparation contains one unit. Therefore, the content of CoA in the preparation will be 1.07 U/mg. and this will henceforth be used as the standard CoA.

TABLE I

Acetylation with Varying Amount of CoA

Amt. of CoA (Crude CoA preparation, *mg.*)	Control Ferricyanide azotometry λ in N_2 (A)	Residual INAH Dichromate azotometry λ in N_2 (B)	Acetyl-INAH λ in N_2 (A—B)
2	9.4	6.7	2.7
4		3.9	5.5
6		2.9	6.5
8		1.8	7.6
10		2.1	7.3
12		2.0	7.4

Acetylation was effected by the method described in the text. CoA used was a dried sample added in amount of 20–30 mg./2 ml.

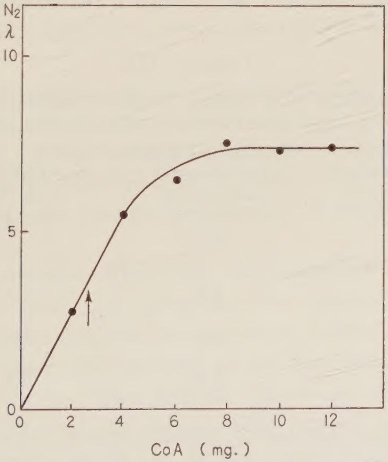


FIG. 2. Acetylation in the presence of varying amount of coenzyme A.

Ordinate indicates the volume of N_2 gas evolved from Acetyl-INAH.

Variation in the amount of CoA used effects changes in the amount of Acetyl-INAH formed and this should result in the change of residual INAH, but ferricyanide azotometry will give the sum of the two compounds.

Experimental results indicated that variations in the amount of CoA has no effect on the results of ferricyanide azotometry (Table II).

TABLE II

Amount of Nitrogen Generated in Ferricyanide Azotometry with Varying Amount of CoA

Amt. of CoA (mg.)	INAH+Acetyl-INAH λ in N_2
0	10.4
5	10.9
10	10.9

CoA, 20 mg./ml. 0.25 or 0.5 ml. used.

b) *Effect of the Amount of Apoenzyme on Acetylation*—Effect of varying the amount of apoenzyme, while the amount of CoA is kept constant (upper limit of 10 mg. taken), on acetylation was examined and the results obtained are shown in Table III and Fig. 3. The maximum rate of acetylation reached 89 per cent. With 10 mg. of CoA, increase of apoenzyme above 0.75 ml. was found to increase the rate of acetylation by 11 per cent as compared with that in the case of 0.75 ml. It may be seen from Table I that 0.75 ml. of apoenzyme with 8 mg. of CoA is able to effect the same rate of acetylation as that with 10 mg. of CoA and this shows that both amounts of CoA have identical effects.

TABLE III

Acetylation with Varying Amount of Apoenzyme

Amt. of apoenzyme	Control Ferricyanide azotometry λ in N_2 (A)	Residual INAH Dichromate azotometry λ in N_2 (B)	Acetyl-INAH λ in N_2 (A—B)	Acetylation rate $(A-B)/A\times 100$
1/2	9.4	5.2	4.2	45
1		2.1	7.3	78
3/2		1.0	8.4	89

Acetylation reaction was carried out with 0.4 ml. of standard CoA (30 mg./1.2 ml.) and 0.375, 0.75, or 1.125 ml. of the apoenzyme.

Comparison of the Present Azotometric Method with the Sulfanilamide Method for Determination of Tissue Coenzyme A—The foregoing experiments have proved that the various conditions for the determination of CoA by azotometry

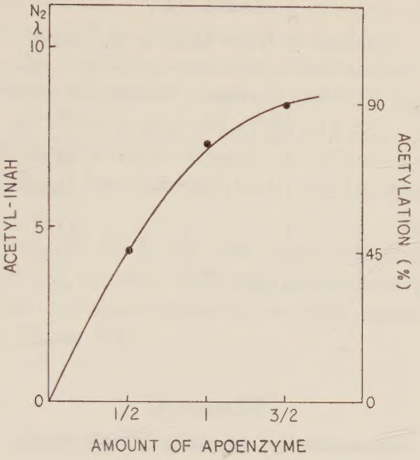


Fig. 3. Effect of varying amount of apoenzyme on acetylation rate of INAH.

are satisfied. The amount of CoA in the rat tissues was determined by the present azotometric method and results were compared with those obtained by the sulfanilamide method carried out at the same time.

The following two experiments were carried out concurrently.

1. The amount of acetylation catalyzed by one unit of previously prepared standard CoA and the amount of saturated acetylation (usually 4 units) were determined by the present azotometry and by the sulfanilamide method.

2. The unknown sample to be evaluated was measured by the two methods at two levels which were around one unit point, slightly below and above it respectively. The reading for the test sample was divided by the reading for the unit of the standard CoA. The number of units in the tissue was calculated from the average value of the volumes of extract containing one unit.

Example of Determination—The CoA contained in the rat tissues was first extracted with water. For this purpose, the albino rat was sacrificed by a blow on the head, the desired tissues were taken out, and chilled in ice. After weighing, the tissue was ground in an ice-cold mortar with a suitable quantity of ice-cold water. The ground tissue was placed in a small beaker of 35×50 mm. capacity and the beaker was heated in a water bath of 100° for 5 minutes. The contents of the beaker were centrifuged and an opalescent supernatant containing CoA was obtained. The volume of water to be added to the tissue should be regulated according to the amount of CoA present and the volume of water added to various tissues in the present work is listed in Table IV.

The extracts were assayed by the INAH method as described above.

In the case of the sulfanilamide method, not exceeding 0.25 ml. of the aqueous solution containing CoA was added to the acetylation reaction

TABLE IV

Amount of Water added to the Tissue

Tissue	Amt. of tissue (g.)	Volume of water added (ml.)
Liver I	1	24.0
Liver II	1	15.0
Kidney	1	11.0
Brain	2	7.0
Heart	0.95	10.5

TABLE V

Determination by Azotometry (INAH Method)

Sample	Amt of CoA (unit)	N_2 gas corresponding to Acetyl-INAH (λ)	Unit	ml/U.	U/g.
Standard CoA	3×4 3×1	7.4 4.3	((4.3+7.4×1/2)×1/2=4.0 4.0 λ correspond to 3 units.)		
Kidney	0.60 ml. 0.45 ml.	4.7 4.0	3×1.18 3×1.0	0.160	75
Standard CoA	3×4 3×1	5.0 2.6	((2.6+5.0×1/2)×1/2=2.6 2.6 λ correspond to 3 units.)		
Liver II	0.50 ml. 0.35 ml.	3.1 2.6	3×1.2 3×1.0	0.126	127

Standard CoA was made into solutions of 2.8 and 11.2 mg./ml. and 0.75 ml. of each solution was used. It had been found by preliminary experiments that 4×3 units effect saturated acetylation, and at this level each measurement. was made.

TABLE VI

Determination by Photometry (Sulfanilamide Method)

Sample	Amt. of CoA (unit)	E	Free sulfanilamide (μg./ml. of react. soln.)	Acetylated sulfanilamide (μg./ml. of react. soln.)	Unit	ml./U	U/g.
Standard CoA	0 1 4	0.611 0.4855 0.380	72.3 57.5 44.9	— 14.8 27.4	((14.8+27.4×1/2)×1/2=14.3 14.3 μg. correspond to 1 unit.)		
Heart	0.25 ml. 0.20 ml.	0.4855 0.503	57.5 59.4	14.8 12.9	1.04 0.90	0.240 0.222	52

Determination followed the method described in the main text.

Standard CoA was made into solutions of 2.8 and 11.2 mg./ml. and 0.25 ml. of each solution was used.

system and the whole volume of the reaction mixture was made up to 1.0 ml. This mixture was incubated at 37° for 2 hours, 4 ml. of 5 per cent TCA were added to stop the reaction, and the supernatant layer was separated by centrifugation. One ml. of the supernatant was taken for determination by the procedure of Bratton and Marshall (*7*). Readings were made on a Rouy Photometer, at a wave length of 535 mμ.

Results obtained from the two methods are indicated in Tables V and VI.

The determination of CoA by the above-described azotometry and photometry was made on entirely different substrates of acetylation. The determinations carried out concurrently on the same tissue extracts gave the results shown in Table VII.

TABLE VII

Comparison of Azotometry and Photometry

Sample	(U/g.) By azotometry	(U/g.) By photometry
Liver I	157	155
Liver II	127	122
Kidney	75	76
Brain	29	28
Heart	56	52

These results show that concurrent determinations of CoA by the two methods give approximately the same values and indicate that the INAH method is effective for the determination of CoA.

It had been previously reported that chicken liver homogenate can effect acetylation of INAH (*5*) and the present work has confirmed the formation of Acetyl-INAH in the enzymic reaction system, as evidenced by paper chromatography.

The control experiments of the present method have shown that there is no consumption of INAH by oxidation or other reactions in this system, and that there is no formation of a substance which might generate nitrogen gas by this azotometry, other than INAH and its derivatives.

SUMMARY

1. An azotometric method for the determination of CoA was established on the basis of the enzymic acetylation of INAH by the function of CoA and specificity of INAH in this azotometry.

2. The characteristics of this method are (i) absence of INAH consumption in the reaction of this system and (ii) absence of substances, other than INAH and its derivatives, which might generate nitrogen gas in this azotometry.

REFERENCES

(1) Kaplan N. A., and Lipmann, F., *J. Biol. Chem.*, **174**, 37 (1948)
(2) Itoh, G., *J. Japan. Biochem. Soc.*, **27**, 145 (1955)
(3) Matsuda, Z., *J. Japan. Biochem. Soc.*, **26**, 420 (1954); *J. Japan. Biochem. Soc.*, **27**, 366 (1955)
(4) Iwasaki, K., and Waseda, M., *J. Japan. Biochem. Soc.*, **28**, 817 (1957)
(5) Itoh, G., *J. Japan. Biochem. Soc.*, **25**, 338 (1953)
(6) Needham, D. M., *Biochem. J.*, **36**, 113 (1942)
(7) Bratton, A. C., and Marshall, E. K. Jr., *J. Biol. Chem.*, **128**, 537 (1939)

The Journal of Biochemistry, Vol. 48, No. 5, 1960

STUDIES ON HISTIDINE RESIDUES IN HEMEPROTEINS RELATED TO THEIR ACTIVITIES

III. PHOTOOXIDATION OF CYTOCHROME C IN THE PRESENCE OF METHYLENE BLUE

By MISAKO NAKATANI

(*From the Department of Agricultural Chemistry, Faculty of Agriculture, Kyushu University, Fukuoka*)*

(Received for publication, January 26, 1960)

It has been suggested from titration data of cytochrome c that two histidines among three per mole are coordinated with a heme iron (*1*). Paléus *et al.* (*2*, *3*) have obtained a peptide bound to the hematin from pepsin digest of cytochrome c and have shown that one histidine residue is present in the peptide. Thus, this histidine was proposed to be linked with the heme.

Using photooxidation technique in the presence of methylene blue, Weil *et al.* studied a histidine-specific oxidation with several proteins (*4*, *5*). The photooxidation of cytochrome c was thus performed, with the view that histidine residues are essential to maintain the coordination structure of heme and might relate to the enzymatic activity of this protein. The results indicated that a complete inactivation of cytochrome c was brought about by the destruction of one of the histidine residues in the molecule. This suggests that the single histidine residue is required for the activity of transfering electrons; possibly the function is maintained by the coordination between heme and imidazole group of this protein.

EXPERIMENTALS

1. Cytochrome c—A crystalline sample of lyophilized cytochrome c (*6*) was supplied through the courtesy of Drs. Okunuki and Sekuzu, Osaka University. Before use, the cytochrome c was oxidized by adding a few drops of 0.01 *M* potassium ferricyanide solution. The concentration of cytochrome c was determined by measuring its optical density at 550 mμ in *M*/30 phosphate buffer (pH 7.4), assuming that the molar extinction coefficient is $\varepsilon_{550} \times 10^{-4} = 0.92\,M^{-1}\,\mathrm{cm}^{-1}$ (*7*).

2. Preparation of Cytochrome c Oxidase—The green brei was prepared by the routine procedure (*8–10*). A fresh bovine heart was minced, washed well with water to remove myoglobin and hemoglobin, homogenated in cold *M*/10 phosphate buffer (pH 7.4) and then centrifuged. The precipitate was washed repeatedly with 2 *M* acetate buffer of pH

---

* Present Address: Research Institute of Angiocardiology, Faculty of Medicine, Kyushu University, Fukuoka.

4.6, then with the addition of 40 mg. of Na-deoxycholic acid per ml. of the green brei solution, the mixture was centrifuged at 25,000×*g* for 60 minutes at 0°. To the solubilized supernatant, crystalline ovalbumin was added and immediately lyophilized. The Q_{O_2} of the preparation thus obtained was about 1,100.

3. *Preparation of Heme*—Protoheme was prepared from bovine erythrocyte (*11*), and was used as pyridine-hemichrome, dissolving in 20 per cent pyridine.

4. *Assay of the Activity of Cytochrome c*—The assay was made with a Warburg manometer at 25° (*12, 13*). The main chamber contained 0.3 ml. of 0.1 *M* semicarbazid-HCl solution of pH 7.1, 0.5 ml. of cytochrome oxidase solution which contained 0.1 mg. of lyophilized sample, 1.45 ml. of 0.1 *M* phosphate buffer (pH 7.4) and 1.0 mg. of cytochrome c or photooxidized cytochrome c in 0.25 ml. of water. From the side arm, 0.25 ml. of 12 mg. per ml. hydroquinone solution was tipped in at the beginning of the reaction. Total volume was adjusted to 3.0 ml. with distilled water. The electron transfering activities of cytochrome c preparations were compared by the O_2 uptake after 30 minutes of the reaction in the mentioned system.

5. *Measurement of O_2 Uptake during the Photooxidation*—The rate of oxygen uptake was determined by the manometric technique which was essentially the same as Weil's method (*14*), using a Warburg manometer. Irradiation with visible light was provided by two 300 W spotlight lamps in parallel over a length of 50 cm. and the lamps were placed at 10 cm. from the glass wall of the water bath and at a distance of 35 cm. from the manometers. The light was reflected onto the Warburg vessels by a mirror which was placed in the water bath at a 45° angle to the incident rays. The main chamber contained 1.0 ml. of cytochrome c solution and 1.0 ml. of 0.2 *M* phosphate buffer. From the side arm, 0.3 ml. of 0.02 per cent methylene blue solution was introduced into the main chamber at the beginning of the reaction. In the center well, a strip of filter paper was placed which was soaked with 0.2 ml. of 15 per cent KOH in order to absorb carbon dioxide, and the total volume was made to 3.0 ml. with distilled water. The reaction was carried out at 30°, and the temperature increase by the irradiation was prevented by placing a glass tube with circulating cold water into the water bath. The temperature of the water bath could be controlled constant when the room temperature was below 20°.

6. *Determination of Amino Acids*—At certain levels of O_2 uptake, each portion of the reaction mixture was taken out and hydrolyzed with 5.5 *N* HCl at 110° for 10 hours. By vaccum distillation, HCl was removed, and colored materials which appeared during the hydrolysis were removed by adding a small amount of charcoal. The volume was then increased to 10 ml. The amount of histidine was determined by Hunter's method (*15*) modified by Narita *et al.* (*16*).

RESULTS

1. *Photooxidation of Cytochrome c at Different pH*—To determine the optimal pH of the photochemical reaction, 1 ml. of aqueous solution containing about 3.7 mg. of cytochrome c was adjusted to pH 5.1, 7.1 or 9.2, respectively, by the addition of a small amount of *N*/10 NaOH or HCl, and placed in the main chamber of a Warburg vessel together with 1.0 ml. of 0.2 *M* acetate or phosphate buffer of the corresponding pH. Then the rate of O_2 uptake was measured (Table I). Oxygen uptake was found to be highest at pH 9.2 and no change in enzymatic activity occured at this pH, so that the experiments were performed thereafter in a phosphate buffer

adjusted to pH 9 with NaOH. Oxygen uptake was also measured without light irradiation or methylene blue. The rate of oxidation, however, was negligibly small.

2. *Photooxidation of Pyridine Hemichrome*—To determine the O_2 uptake of hematine part, pyridinehemichrome solutions of 1.0, 0.5 and 0.1 mg. of protoheme in 1 ml. of 20 per cent pyridine were photooxidized under the same conditions as described above. However, no oxygen consumption was observed for heme or pyridine at any pH tested. The oxygen uptake in the presence of cytochrome c under the conditions described above would be attributed to the photochemical oxidation of the protein moiety of cytochrome c where most probably histidine and other aromatic amino acid residues are oxidized.

TABLE I

Effect of pH on the Rate of Oxygen Uptake of Cytochrome c during the Photooxidation

Time (min.)	pH of the reaction Mixture		
	9.2	7.1	5.1
	(μl.)		
10	11.5	2.7	1.0
20	19.2	5.6	1.9
30	27.3	8.5	2.8
60	39.0	14.2	5.3
90	43.8	18.4	7.8
120	47.0	20.5	8.7

3. *Decrease in Activity and Amino Acids Contents of Cytochrome c during Photooxidation*—The cytochrome c solution of 7.4 mg. per ml. concentration was photooxidized at pH 9.0. Upon 30, 60, 90 and 180 minutes of irradiation, 29.0, 46.5, 58.5 and 79.0 μl. of oxygen were respectively consumed. These values of oxygen consumed correspond to 2.1, 3.4, 4.2 and 5.7 moles O_2 per moles of cytochrome c on the basis that the molecular weight of cytochrome c is 12,000.

Samples after different durations of irradiation were analyzed for the enzymatic activity and for contents of histidine and tyrosine (Fig. 1).

With the intact cytochrome c, analytical data of these amino acid contents were: histidine, 3.9 per cent (3.0 moles) and tyrosine, 5.5 per cent (3.7 moles). The calculations were made, assuming that the molecular weight and N content of cytochrome c are 12,000 and 15.36, respectively (*7*, *17*).

4. *The Absorption Spectra of Photooxidized Cytochrome c*—Cytochrome c solutions were photooxidized under the same conditions as described above.

After 60 and 120 minutes, irradiation, methylene blue was removed from each of the solutions by the addition of a small amount of charcoal, and

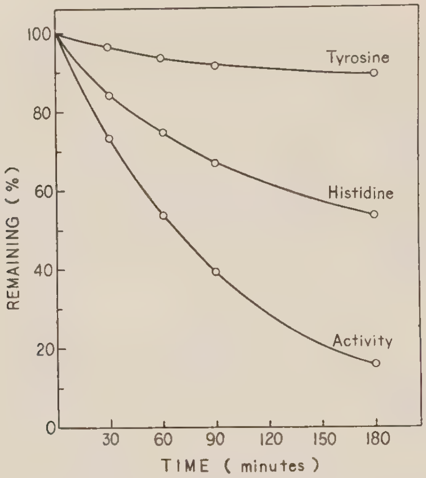


Fig. 1. Effect of irradiation time on histidine and tyrosine contents and enzymatic activity of cytochrome c.

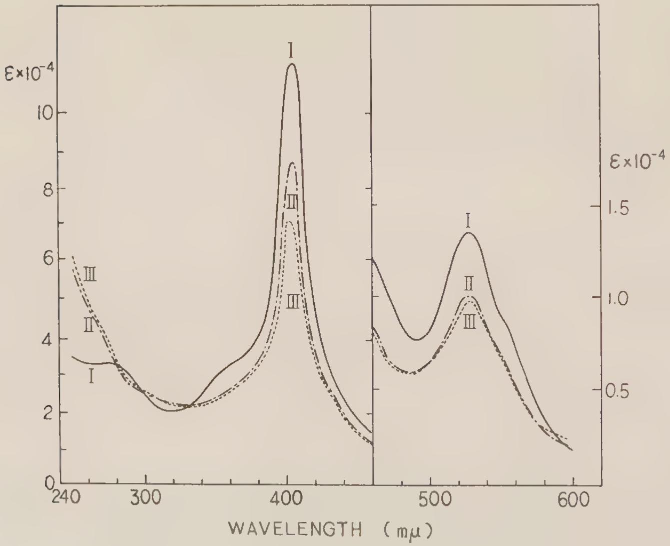


Fig. 2. Effect of photooxidation on the absorption spectrum of cytochrome c. Curve I, Native; Curve II, after 60 min. photooxidation; Curve III, after 120 min. photooxidation.

the absorption spectra of the remaining solutions were measured (Fig. 2). The absorptions at 500 mμ to 600 mμ and 400 mμ were remarkably decreased.

Since the absorption in the Soret region is assumed to be partly dependent on the mode of linkage between heme and protein (discussed in a previous paper (*18*)), and since no oxygen consumption attributable to the photooxidation of porphyrin was observed, the decrease in absorption in this region by photooxidation might have resulted from the modification of the protein configuration; probably histidines linked with heme were destroyed, which might have resulted in the decrease in absorption. The absorbancy change around 280 mμ might have been due to the destruction of tyrosine and tryptophan during photooxidation.

DISCUSSION

Using the photooxidation technique, the relation between the enzymatic activity and histidine contents of cytochrome c was investigated. No exact relationship, however, could be demonstrated. The studies with chymotrypsin and ribonuclease revealed that the complete inactivation resulted from the destruction of one mole of histidine per mole of each enzyme (*4*, *5*). Also it was found by Koshland *et al.* (*19*) that only one of the histidines was required for enzymatic activity of phosphoglucomutase, when the equation, $A/Ao=H/Ho$, could be held (A, Ao: enzymatic activity at photooxidation time t and O, and H, Ho: histidine content at t and O). In the present study, complete inactivation of cytochrome c was reached by the loss of 43 per cent of histidine (1.3 moles per mole), so that the kinetic analysis by Koshland *et al.* was applied to the results of cytochrome c. For the analysis, the following assumption was made: (i) one mole of histidine was required for enzymatic activity, since complete inactivation was reached by the destruction of less than 2 moles of histidine, and (ii) the rate of overall destruction of histidine as observed was the sum of two different destruction rates of histidines of different susceptibilities, *i.e.*, "fast" and "slow" destructable groups, since a log plot of the total decrease in histidine against time was not linear (Fig. 3). The "fast" group which was assumed to be required for the activity was taken to be one mole, and two moles were assumed to belong to the "slow" group, according to the method of Koshland *et al.*

The linear relationship between log plots of the rates of inactivation and histidine destruction was obtained by calculation. The results showed that the remaining enzymatic activity corresponded to the remaining histidine in the "fast" group. According to Koshland *et al.*, this relationship is held only when the "fast" destructable group of histidine alone is required for the enzymatic activity.

The rate of destruction of the "fast" group was more than 7 times higher than that of the "slow" group. Since cytochrome c is a rigid protein and a heme group is considered to be imbeded, it might be an effect of neighbouring groups that led to an easy destruction of "fast" histidine.

With respect to the structure of cytochrome c, two histidines have been assumed to be coordinated with the heme iron from each side of the plane of the porphyrin ring (20). If both of two histidines were required for the enzymatic activity, the relationship between the enzymatic activity and photooxidized histidine might have had a higher degree of correlation, such as $A/Ao=(H/Ho)^2$. However, the results showed that only one histidine is required for the enzymatic activity. It appears that the cytochrome c is no longer active after the destruction of one of the coordinated histidines, or only one of the histidines is required to maintain the active structure around heme-protein linkage, which is essential to the enzymatic activity.

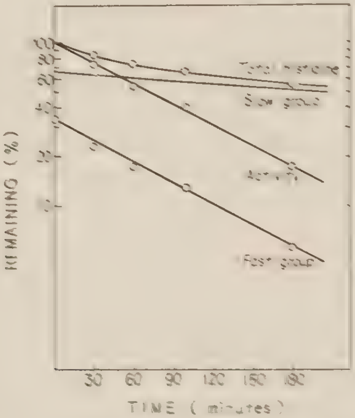


Fig. 3. Effect of irradiation on histidine content and enzymatic activity of cytochrome c.

Destruction of histidine was analyzed in terms of 2 straight lines of "slow" and "fast" destrucable histidine groups.

It was observed that about 5 to 6 moles of O_2 were taken up until the complete inactivation of cytochrome c was reached by photooxidation. This suggests that also other amino acids than histidine should have been oxydized. These groups might be tyrosine, tryptophan, cysteine and methionine as reported with other proteins. Since it was only 20 per cent of total tyrosine that was destroyed (0.7 moles per mole of cytochrome c), about 3 to 4 moles of other amino acids might have been photooxidized. Those amino acids other than histidine may possibly be required directly or indirectly for the enzymatic activity. However, the considerable agreement between the rates of inactivation and destruction of the "fast" group of histidine seems to indicate the presence of a direct relationship between the enzymatic activity and histidine.

SUMMARY

1. Cytochrome c was photooxidized in the presence of methylene blue.

Its enzymatic activity and histidine content were decreased during the reaction.

2. The complete inactivation was brought about by a loss of 43 per cent of total histidine which corresponded to 1.3 moles per mole of cytochrome c.

The kinetic analysis of the reaction revealed the presence of two kinds of histidine residues in terms of their susceptibilities to the photooxidation: "slow" destructable and "fast" destructable groups. Also it was suggested that only the "fast" destructable histidine is required for the activity of cytochrome c.

3. The absorption spectrum was modified considerably by photooxidation, and the decreases in absorbancy at 500 to 600 mμ and 404 mμ were particularly significant.

The author wishes to thank Prof. K. Okunuki and Dr. I. Sekuzu, Osaka Univ., for kindly supplying 'crystalline cytochrome c', and to express her appreciation for the invaluable advice and criticism of Prof. Y. Oshima and Prof. M. Funatsu, Kyushu Univ., during the course of this work.

REFERENCES

(*1*) Wyman, J., *J. Biol. Chem.*, **127**, 1 (1939)
(*2*) Paléus, S., Ehrenberg, A., and Tuppy, H., *Acta Chem. Scand.*, **9**, 365 (1955)
(*3*) Paléus, S., Ehrenberg, A., and Tuppy, H., *Acta Chem. Scand.*, **9**, 353 (1955)
(*4*) Weil, L., James, S., and Buchert, A. R., *Arch. Biochem. Biophys.*, **46**, 266 (1953)
(*5*) Weil, L., Seibles, T. S., *Arch. Biochem. Biophys.*, **54**, 365 (1955)
(*6*) Hagihara, B., Morikawa, I., Sekuzu, I., and Okunuki, K., *J. Biochem.*, **45**, 551 (1958)
(*7*) Margoliash, E., *Biochem.* J., **56**, 529,535 (1954)
(*8*) Wainio, W. W., Cooperstein, S. J., Kollen, S., and Eichel, B., *J. Biol. Chem.*, **173**, 145 (1948)
(*9*) Eichel, B., and Wainio, W. W., *J. Biol. Chem.*, **175**, 155 (1948)
(*10*) Smith, L., and Stotz, E., *J. Biol. Chem.*, **209**, 819 (1954)
(*11*) Willstätter, R., *Ann.* **373**, 232 (1910); *Ann. Chem.*, **385**, 197 (1911)
(*12*) Eichel, B., Wainio, W. W., Person, P., and Cooperstein, S. J., *J. Biol. Chem.*, **183**, 89 (1950)
(*13*) Dannenberg, H., and Kiese, M., *Biochem. Z.*, **322**, 395 (1952)
(*14*) Weil, L., and Maher, *Arch. Biochem.*, **29**, 241 (1950)
(*15*) Hunter, A., *J. Biol. Chem.*, **196**, 589 (1952); *J. Biol. Chem.*, **216**, 391 (1955)
(*16*) Narita, K., and Okada, Y., "Chemistry of Proteins," Kyoritsu Shuppan Co., Ltd., Tokyo, Vol. **1**, p 238 (1954)
(*17*) Theorell, H., Akeson, A., *J. Am. Chem. Soc.*, **63**, 1804 (1941)
(*18*) Nakatani, M., *J. Biochem.*, **48**, 476 (1960)
(*19*) Koshland, D. E. Jr., Ray, W. J. Jr., and Erwin, M. J., *Federation Proc.*, **17**, 1145 (1958)
(*20*) Margoliash, E., *Nature.* **175**, 293 (1955)

The Journal of Biochemistry, Vol. 48, No. 5, 1960

STUDIES ON HISTIDINE RESIDUES IN HEMEPROTEINS RELATED TO THEIR ACTIVITIES

IV. PHOTOOXIDATION OF PEROXIDASE IN THE PRESENCE OF METHYLENE BLUE

By MISAKO NAKATANI

*(From the Department of Agricultural Chemistry, Faculty of Agriculture, Kyushu University, Fukuoka)**

(Received for publication, January, 26, 1960)

Histidine residues in hemeproteins such as cytochrome c and hemoglobin have been believed to be the sites of binding of heme iron. However, in peroxidase they have been assumed to be unrelated to the linkage between heme and protein (*1*).

As reported in the preceding paper of this series (*2*), it has been observed that the photooxidation of cytochrome c in the presence of methylene blue resulted in a complete loss of enzymatic activity which seemed to be accounted for by the destruction of one of the histidine residues in the molecule.

In order to obtain additional information as to the relationship between the activity of hemeprotein and histidine residues, the effects of photooxidation on the enzymatic activity of peroxidase have been investigated.

EXPERIMENTAL

1. Peroxidase Sample—Crystalline japanese radish peroxidase (*3*) was kindly supplied by Dr. Morita, Kyoto Univerity. The crystalline peroxidase in 0.70 saturated ammonium sulfate solution was dissolved in water, dialyzed against distilled water for a week, replacing the outer solution several times, at 0–5° and used for the experiment. The concentration of peroxidase was determined by absorbancy measurement of the dialyzed solution at 276 mμ regarding its extinction coefficient as $\varepsilon_{276} \times 10^{-4} = 3.38$, and its molecular weight as 55,000 (*3*).

2. Assay of the Peroxidase Activity—The activity was assayed by the guaiacol test according to the method of Maehly and George (*4*, *5*). One ml. of 20 m*M* aqueous guaiacol solution and 2.0 ml. of 10 m*M* phosphate buffer of pH 7.0 were placed in an optical cell of 1.0 cm. light path, together with an appropriate amount of the enzyme solution (0.01 to 0.05 ml). To the reaction mixture, 20 μl. of 10 m*M* H_2O_2 were added, and the time required to reach 0.050 optical density unit at 470 mμ was measured by a Beckman model DU spectrophotometer. For routine measurements of activity, the final enzyme concentration was 0.001 to 0.003 mg. at which the time required to reach the unit was between 10 to 30 seconds. The rate of product formation (tetraguaiacol), dx/dt, was computed by the following equation, using the milimolar extinction coefficient 26.6, at 470 mμ, for tetraguaiacol:

---

* Present Address. Research Institute of Angiocardiology, Faculty of Medicine, Kyushu University, Fukuoka.

$$dx/dt = 1.88 \times 10^{-6}\,M \text{ tetraguaiacol} \times 1/t.$$

RESULTS

1. *Oxygen Uptake during the Photooxidation*—The rate of O_2 uptake was measured by the manometric technique at 30°, using a Warburg manometer as reported previously (*2*). The reaction mixture in the main chamber consisted of 1.0 ml. peroxidase solution containing approximately 9.2 mg. of the dialyzed sample adjusted to pH 9.0 with $N/10$ NaOH, 1.0 ml. of 0.2 M phosphate buffer of pH 9.0, 0.3 ml. of 0.02 per cent methylene blue solution and 0.5 ml. of water. The center well contained 0.2 ml. of 15 per cent KOH and a strip of filter paper. The results are shown in Table 1.

TABLE I

O_2 Uptake of Peroxidase during the Photooxidation

(9.2 mg. of peroxidase was used, and its molecular weight was taken as 55,000).

Time (min.)	O_2 uptake	
	μl.	mole[1]
10	4.0	1.1
20	6.7	1.8
30	9.4	2.5
40	12.0	3.2
50	14.5	3.8
60	16.8	4.5
90	22.4	5.9
120	27.0	7.2

1) moles of oxygen consumed per mole of peroxidase.

2. *Determination of Histidine Content During the Photooxidation*—At the end of the above photooxidation experiment, the reaction mixture was taken from the Warburg manometer vessel, and used for the two separate experiments—measurement of absorption spectrum and determination of histidine content. From the reaction mixture, containing 9.2 mg. peroxidase in 2.8 ml., 2 ml. was taken and hydrolyzed with 5.5 N HCl at 110° for 10 hours. The histidine content was determined as previously reported, using the method of Hunter (*6*) modified by Narita *et al.* (*7*). The N concentration in the hydrolyzed solution was determined by the micro-Kjeldahl method, and histidine content was calculated, assuming that the N content

of peroxidase is 12.73 per cent (*3*). At the same time, intact peroxidase was also hydrolyzed and the histidine content was determined as a blank. The analytical value of histidine content of intact peroxidase was 1.1 per cent, which is in agreement with the value reported by Morita (*8*). On the other hand, histidine centent of the photooxidized peroxidase was less than 0.15 per cent. This means that about 85 per cent of histidine residues in the peroxidase molecule (corresponding to 3.4 moles in 4 moles of total histidine per mole of peroxidase) was destroyed by the photooxdation.

3. Determination of the Absorption Spectrum—A portion of the photooxidized reaction mixture was treated with a small amount of charcoal to remove methylene blue, and the absorption spectrum was measured after being diluted with $M/10$ phosphate buffer of pH 6.8 (the final concentration of the buffer was adjusted to $M/40$) (Fig. 1).

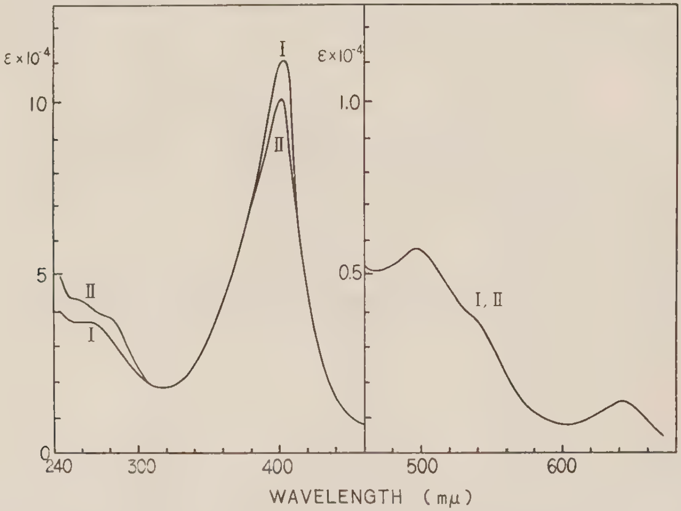


Fig. 1. Effect of photooxidation on the absorption Spectrum of Peroxidase.

Curve I, Native; Curve II, Photooxidized for 120 min.

4. Activity Change of Peroxidase during the Photooxidation—The reaction mixture which contained 0.48 mg. peroxidase in the final volume of 2.8 ml. was photooxidized under the same conditions as described for the measurement of oxygen uptake. (The composition of the reaction mixture was the same as above). At 30-minute intervals of the irradiation, aliquots of the reaction mixture were taken and the enzymatic activity was measured (Table 2).

TABLE II

Activity Change of Peroxidase during the Photooxidation

In the reaction mixture of photooxidation, 0.17 mg. of peroxidase per ml. were contained, and 0.01 ml. of this solution was used for the assay.

Time[1] (min.)	t[2] (sec.)	$1/t$	$dx/dt \times 10^{-7}$
0	15.0	0.0667	1.254
30	15.0	.0667	1.254
60	15.1	.0662	1.244
90	15.1	.0662	1.244
120	15.2	.0658	1.237

1) Time of irradiation.

2) Time required for reaching 0.050 optical density unit at 470 mμ.

DISCUSSION

As reported previously, the photooxidation of cytochrome c resulted in a complete loss of enzymatic activity and a destruction of only one of the histidine residues in the molecule. Hence it was assumed that this histidine residue of cytochrome c which was accessible to the photooxidation was responsible for the enzymatic activity (*2*). The results with peroxidase showed, however, that there was only little loss of enzymatic activity even when 85 per cent of the histidine residues had been destroyed by photooxidation. This seems to indicate that the histidine residues in peroxidase have no direct relation to its enzymatic activity.

Also the experimental finding that the change of the absorption spectrum of peroxidase was slight even after the extensive photooxidation of histidine residues seems to support that histidine residues do not contribute significantly to the absorbancies in the visible region. On the other hand, the absorption spectrum of cytochrome c changed remarkably after photooxidation, and this protein has been considered to have a structure in which histidines coordinate with heme-iron. These facts, together with those obtained in this study appear to suggest that the absorptions of hemeproteins either in the visible region and particularly in the Soret region may partly be dependent on the mode of linkage between heme and protein, as discussed previously in this series of report (*9*).

SUMMARY

1. Crystalline japanese radish peroxidase was photooxidized in the presence of methylene blue. The enzymatic activity remained without a detectable loss when 85 per cent of the total histidine residues in the molecule was lost.

2. By photooxidation of the peroxidase, only a slight decrease in the

absorbancy in the visible region was observed.

The authour wishes to thank Dr. Y. Morita, Kyoto University, for kindly supplying crystalline japanese radish peroxidase, and to express her appreciation for the invaluable advice and criticism of Prof. Y. Oshima and Prof. M. Funatsu, Kyushu University, during the course of this work.

REFERENCES

(*1*) Morita, Y., *Bull. Research Inst. Food Sci., Kyoto Univ.*, **15**, 64 (1954)

(*2*) Nakatani, M., *J. Biochem.*, **48**, 633 (1960)

(*3*) Morita, Y., and Kameda, K., *Memories Research Inst. Food. Sci., Kyoto Univ.* **12**, 14 (1957)

(*4*) Maehly, A. C., "Method of Biochemical Analysis," Interscience Publishers, Inc., New York, Vol. **1**, p. 386 (1957)

(*5*) George, P., *J. Biol. Chem.*, **201**, 413 (1953)

(*6*) Hunter, A., *J. Biol. Chem.*, **196**, 589 (1952), *J. Biol. Chem.*, **216**, 391 (1955)

(*7*) Narita, K., and Okada, Y., "Chemistry of Proteins," Kyoritsu Shuppan Co., Ltd., Tokyo, Vol. **1**, p. 238 (1954)

(*8*) Morita, Y., and Kameda, K. *Bull. Agr. Chem. Soc.*, **23**, 28 (1959)

The Journal of Biochemistry, Vol. 48, No. 5, 1960

IMMUNOCHEMICAL STUDIES OF INSULIN

III. THE IMMUNOLOGICAL ACTIVITIES OF INSULIN DERIVATIVES

By MASAYASU KITAGAWA, KAORU ONOUE, MOTOAKI ANAI
AND YUICHI YAMAMURA

*(From the Department of Biochemistry, Faculty of Medicine,
Kyushu University, Fukuoka)*

(Received for publication, February 8, 1960)

Chemical modification of protein has been attempted by many investigators to determine the specific groups responsible for antigen-antibody reaction. In such a research, insulin is considered to be a suitable protein as an antigen, since it has a biological activity and its chemical structure is well known already. However, very little has been made concerning such an experiment, for the reason that it has been difficult to obtain the clearly demonstrable antibodies against insulin.

Recently, Grodsky, Pegs and Forsham (*1*) demonstrated that some modified insulin competed with insulin-I^{131} for the binding site of insulin binding antibodies existed in the serum of insulin resistant subjects, using hydrodynamic flow technique.

It was previously shown by the present authors that the insulin neutralizing antibodies and skin allergic antibodies in guinea pigs were obtained (*2–4*).

In the present study, the interaction between various insulin derivatives and these insulin antibodies was investigated in order to approach the information concerning chemical mechanisms of antigen-antibody reaction.

EXPERIMENTALS

Preparation of Antiinsulin Sera—Guinea pigs were immunized with ox insulin according to the method as previously described (*2, 3*).

The insulin neutralizing activity of antiserum was assayed by mouse convulsion procedure (*2, 3*).

Preparation of Insulin Derivatives—Whale insulin preparation was kindly supplied by Dr. T. Sawada, 3rd Department of Internal Medicine, Faculty of Medicine, Kyushu University and Dr. T. Shibata, Taiyo Fishery Co., Ltd.

Esterification was performed by the method of Fraenkel-Conrat (*5*) as follows. One hundred mg. of insulin was suspended in 100 ml. of methanolic hydrochloride (0.1 *N*) and vigorously stirred in a room temperature for 24 hours. Two hundred ml. of ethylacetate was added to the reaction mixture and centrifuged at 1500 r.p.m. Resulting precipitate was washed by 10 ml. of ethylacetate three times and dried *in vacuo*.

Acetylation was carried out by the method of Olcott and Fraenkel-Conrat (*6*), using acetic anhydride as a reagent but pH was not corrected during the course of reaction, final pH being 4–5.

Dinitrophenyl and iodo-insulin were prepared according to the method described by Sanger (*7*) and Harrington (*8*) respectively.

Oxidation of insulin with performic acid was performed by the application of the method of Hirs *et al.* (*9*) applied to ribonuclease oxidation.

In order to give its phenylthiocarbamyl derivative, insulin was treated with phenylisothiocyanate according to the method of Fraenkel-Conrat (*5*).

Palmytoylinsulin was prepared according to the method of Tanaka (*10*), using palmityl chloride.

RESULTS

The various protein reagents employed for the preparation of insulin derivatives are shown in the first column of Table I. Amount of each reagent used for the modification is expressed as equivalent per 6×10^3 g. insulin and is shown in parenthesis.

It was found that acetic anhydride reacted with 98.5 per cent of the original amino groups (colorimetric determination by ninhydrin reaction) (*11*) and also with 25 per cent of the original phenolic groups (Herriot's method) (*12*). Phenylisothiocyanate reacted with 39 per cent of the original amino groups and with 7 per cent of the original phenolic groups.

Denitrofluorobenzen reacted with 99.2 per cent of the original amino groups.

Palmytil chloride reacted with 10 per cent of the original amino groups in case of sample no. 7 and with 16 per cent in case of sample no. 8 respectively. Sample no. 7 was soluble in water but no. 8 insoluble even in alkaline water pH 9. DNP-insulin also was insoluble in water. These insoluble samples were employed as suspension when the chemical analysis or biological activities were examined.

When the iodination was performed under the present condition, all tyrosines of insulin converted to diiodotyrosine (*13*).

The data concerning esterification will be reported elsewhere in detail.

The oxidative product by performic acid has not been enough characterized yet.

Hormonal Activity—Their hormonal activities were assayed by mouse convulsion test according to the method described by Fraenkel-Conrat (*5*).

Results of the hormonal activities of derivatives are shown in the second column of Table I expressed as activity per cent of the original. The treatment of insulin with performic acid, methanolic hydrochloric acid, iodine, dinitrofluorobenzene and excess palmityl chloride caused extensive loss of activity. Considerable inactivations were also obtained upon the reaction with phenylisothiocyanate and acetic anhydride.

Analytical data showed that acetylation by acetic anhydride occurred not only on the amino groups of insulin (98.5 per cent) but also on phenolic groups (25 per cent) under the present condition as above described. When insulin was treated with smaller amounts of palmityl chloride (2.35 equivalent per 6×10^3 g. insulin), the activity was fully retained, although the treatment with excess of palmityl chloride caused the complete loss of the activity.

TABLE I

Immunological Activities of Insulin Derivatives

Derivative No.	Reagent	Hormonal activity per cent or original	Absorption test	Skin test
1	Performic acid	< 1	−	−
2	0.1 *N* Methanolic HCl	< 1	+	+
3	Acetic anhydride (85)	< 8	+	++
4	Iodine (10)	< 1	+	++
5	Phenylisothiocyanate (190)	< 4	+	++
6	Dinitrofluorobenzen	< 1	−	−
7	Palmityl chloride I (2.35)	100	/	+++
8	,, VI (50)	< 1	−	−
9	Non-treated	100	/	+++

Amount of each reagent was expressed as equivalent per 6×10^3 g. insulin, in parenthesis. Hormonal activity of each derivative was assayed by mouse convulsion test. Absorption test: Mice were injected intraperitoneally with the mixture of 0.3 ml. of immune serum and 0.2 ml. of saline containing each derivative and simultaneously with 0.25 units of whale insulin subcutaneously. Amount of each derivative was corresponding to the following unit of original insulin.

No. 1, 2, 4, 6, 8 sample (3 units), No. 3 sample (0.7 units), No. 5 sample (1 unit); + Convulsion occurred − not occurred. Skin test; One tenth ml. of saline containing each derivative corresponding to 0.5 u. of the original insulin was injected to immunized guinea pig intradermally. The size of erythema was measured 2 hours after injection.

+ 5–9 mm, ++ 10–20 mm, +++ over 20 mm in diameter, − no reaction.

Absorption Test—In order to investigate whether the inactivated derivatives reacted with insulin neutralizing antibodies or not, absorption test was devised as follows. As a preliminary experiment, two injections were made into starved mice simultaneously: one injection with mixture of 0.25 ml. of immune serum and 0.25 ml. of buffered saline intraperitoneally, the other one with 0.25 ml. of saline containing 0.25 units insulin subcutaneously. The immune serum used here showed one unit insulin neutralizing activity per ml. by mouse convulsion test. All of ten mice tested did not cause convulsion shock.

Next, 0.3 ml. of the same immune serum was mixed with 0.25 ml. of buffered saline containing each derivative. The amounts of oxidized-, esterified-, iodinated-, DNP- and palmytoyl-VI-insulin corresponded to 3 units of the original insulin, acetylinsulin to 0.7 units and phenylthiocarbamyl-insulin to 1 unit, respectively. Each mixture was injected intraperitoneally and simultaneously 0.25 ml. of saline containing 0.25 units insulin was injected subcutaneously in each group of five mice.

As shown in the third column of Table I, all the mice of groups injected with esterified-, iodinated-, phenylthiocarbamyl- and acetylated-insulin caused the convulsion shock. On the other hand, others did not cause the convulsion in all the mice of each group. These results suggested that the former derivatives seemed to have the ability of combination with insulin neutralizing antibodies though they have lost hormonal activity expressed by the convulsion due to a hypoglycaemic effect. This fact was also confirmed by the following experiment.

As previously described (*4*), the injection of guinea pig immune serum to mice caused marked elevation of blood sugar level. If the insulin neutralizing antibodies combine with the inactivated insulin derivative, the elevation of blood sugar level would not occur by the injection of the mixture of immune serum and insulin derivative. Typical data were shown in Table II, demonstrating that the mixture of immune serum (1 unit/ml.) and insulinester (corresponding to 4 units of the original insulin) did not cause the elevation of the blood glucose level, though the immune serum alone caused marked hyperglycaemia due to neutralization of endogeneous insulin of mice.

TABLE II

Inhibitory Effect of Insulinester on Elevation of Blood Sugar Level Caused by Injection of Immune Serum to Mice

Intraperitoneal injection with	Blood sugar level 2 hr. after injection
Immune serum 0.5 ml. + Buffered satine 0.2 ml.	259±27 mg./dl.
Insulinester 0.2 ml. (4 u.) + Buffered saline 0.5 ml.	81±15
Immune serum 0.5 ml. + Insulinester 0.2 ml. (4 u.)	105±28
Non-treated	86±16

A group of 5 mice was injected intraperitoneally with immune serum alone, insulinestar alone and mixture of immue serum and insulinester, respectively. Mice were sacrificed and blood sugar level was determined after 2 hours. Prior to the test, mice were fasted for 12 hours.

Skin Test—Other immunochemical property of each derivative was tested by a skin reaction. According to the method previously described (*4*), guinea pigs immunized were injected intradermally with 0.1 ml. of saline containing each amount of the derivative corresponding to 0.5 units of original insulin. The sizes of erythema and induration were measured 3 hours after injection. As shown in the fourth column of Table I, other preparations than oxidized-, DNP- and palmytoyl-VI-insulin were positive.

In these results, it was found that some of the inactivated insulin derivatives were immunologically active and there exists the parallel relationship between their absorption test and skin test.

DISCUSSION

In our present report, it was demonstrated that some insulin derivatives, which lost the hormonal activity, were still immunologically active. This may suggest that the active site of insulin as a hormone differ from its binding site in the immunological reaction. In the further investigation, it will be expected to clarify the role of the chemical groups in antigen-antibody reaction, if insulin, the best characterized protein, will be used as an antigen.

On the other hand, one of the difficulties of immunization with insulin is considered to be owing to the following reasons, that is, the injection with considerable amounts of insulin cause often the death of animals to be immunized due to hypoglycaemic shock as reported by Moloney and Goldsmith (*14*). However, the employment of hormonally inactive but immunologically active derivatives will be able to exclude such a difficulty. In fact, we could obtain the considerably active insulin neutralizing antibodies by immunization with insulin ester and iodoinsulin in Freund complete adjuvant, which will be reported elsewhere.

SUMMARY

1. Insulin derivatives were tested immunologically, using the absorption test and skin reaction.

2. Oxidized-, dinitrophenylated- and largely acylated-insulin lost not only the hormonal activity but the immunological activity.

3. Esterified-, acetylated-, iodinated- and phenylthiocarbamyl-insulin lost the hormonal activity but were still immunologically active.

The authors are very grateful to Dr. Y. Nojima, the Kodama Shoji Co., Dr. T. Sawada, 3rd Department of Internal Medicine, Faculty of Medicine, Kyushu University, Dr. T. Shibata, Taiyo Fishery Co., Ltd. and Dr. N. Okuyama, Department of Chemistry, Faculty of Science, Tokyo prefectural University, for gifts of insulin samples. We are also indebted to Dr. M. Yamaguchi, Research Laboratory of the National Sanatorium, Toneyama Hospital for preparation of heat killed tubercle bacilli in the series of these studies.

REFERENCES

(*1*) Grodsky, G. M., Peng, C. T., and Forsham, P. H., *Arch. Biochem. Biophys.*, **81**, 264 (1959)

(*2*) Yamamura, Y., Kitagawa, M., and Onoue, K., *J. Biochem.*, **45**, 947 (1958)
(*3*) Kitagawa, M., Onoue, K., Okamura, Y., Anai, M., and Yamamura, Y., *J. Biochem.*, **48**, 43 (1960)
(*4*) Kitagawa, M., Onoue, K., Okamura, Y., Anai, M., and Yamamura, Y., *J. Biochem.*, **48**, 483 (1960)
(*5*) Fraenkel-Conrat, J., and Fraenkel-Conrat, H., *Biochim. et Biophys. Acta*, **5**, 89 (1950)
(*6*) Olcott, H. S., and Fraenkel-Conrat, H., *Chem. Rev.*, **41**, 151 (1947)
(*7*) Sanger, F., *Biochem. J.*, **39**, 507 (1945)
(*8*) Harrington, C. R., and Neuberger, A., *Biochem. J.*, **30**, 809 (1936)
(*9*) Hirs, C. H. W., Moore, S., and Stein, W. H., *J. Biol. Chem.*, **219**, 623 (1956)
(*10*) Yamamura, Y., Tanaka, A., and Kato, M., *J. Biochem.*, **47**, 505 (1960)
(*11*) Rosen, H., *Arch. Biochem. Biophys.*, **67**, 10 (1957)
(*12*) Heriott, R. M., *J. Gen. Physiol.*, **19**, 283 (1935)
(*13*) Roche, J., Michel, R., and Lafon, M., *Biochim. et Biophys. Acta*, **1**, 453 (1947)
(*14*) Moloney, P. J., and Goldsmith, L., *Canad. J. Biochem. Physiol.*, **35**, 79 (1957)

The Journal of Biochemistry, Vol. 48, No. 5, 1960

ACTION OF CHYMOTRYPSIN ON SYNTHETIC SUBSTRATES

IV. ACTION OF α-CHYMOTRYPSIN ON ACYL-L-TYROSYLGLYCINES AND ACYL-L-TYROSYLGLYCYLGLYCINES*, **

By TAKESHI YAMASHITA***

(*From the Laboratory of Biochemistry, Faculty of Science,*
Kyushu University, Fukuoka)

(Received for publication, February 8, 1960)

In the previous studies, the rates of α-chymotrypsin-catalyzed hydrolysis of synthetic substrates of the α-aminoacyl-L-tyrosine amide and ethyl ester type were reported and the effect of varing the aminoacyl group on the susceptibility of tyrosyl linkage to chymotrypsin was shown (*1–3*).

Earlier studies on this enzymatic action have shown that it was difficult to compare the effect when the acyl group in the acylamino acid derivatives was varied (*4*). The comparison was based not only on the structural effect of substrate but also on the effect of various conditions used in the experiments.

The present paper deals with detailed studies concerning the quantitative effect of varying the acyl group and another moiety (amide, glycine and glycyl-glycine) in acyl-L-tyrosine derivatives. In this study, acyl-L-tyrosylglycine and acyl-L-tyrosylglycylglycine, in which the acyl group is benzoyl, acetyl, glycyl, carbobenzoxy, formyl, *etc.*, were prepared and subjected to the action of chymotrypsin.

EXPERIMENTAL

Enzyme and Methods—The crystalline α-chymotrypsin was the same as that previously used (*1*). Enzymatic hydrolysis of acyl-L-TyrGlyGly, -L-TyrGly or -L-TyrAm was carried out at 30° in 0.1 *M* phosphate buffer (pH 7.8). The method for measuring amidase activity has already been described (*1*). The rate of hydrolysis of the peptide bond was measured and calculated by the colorimetric ninhydrin method (*2*, *5–7*). The color yield of amino acids and peptides, taking that of L-leucine as 100 per cent, is shown in Table I.

In addition to the foregoing measurements, the reaction mixture was analyzed by peper chromatography with the use of butanol-acetic acid-pyridine-water (30 : 6 : 20 : 24

---

* The present paper was read in part before the 12th Annual Meeting of the Chemical Society of Japan (Kyoto, April, 1959).

** The following abbreviations are used: Cbz, carbobenzoxy; Ac, acetyl; Bz, benzoyl; Form, formyl; OEt, ethyl ester; OCH_2Ph benzyl ester; Am, amide; DMF, dimetylformamide.

*** Present address: Asahi Chemical Industries Co., Ltd., Technical Research Laboratory, 1555 Shimura Nakadai, Itabashi-ku, Tokyo by vol.) as the solvent system (Table I). The results indicated that only a specific hydrolysis of the tyrosyl linkages occurred.

TABLE I

R_f Value and Per Cent Color Yield[a]

Substance	Color yield (per cent[b])	R_f Butanol: acetic acid: pyridine: water (30:6:20:24 by vol.)
Gly [c]	100	0.25
GlyGly [c]	89	0.22
Gly-L-Tyr [c]	93	0.50
Gly-L-TyrGly	93	0.44
Gly-L-TyrGlyGly	86	0.40

a) Experimental details are described in the literature (*5*, *8*).

b) Based on L-leucine as 100 per cent.

c) The data are from previous reports (*7*, *8*).

Synthesis of Peptide Derivatives—Coupling of acylamino acid with amino acid esters or peptide esters was accomplished through mixed anhydrides with *iso*-butyl carbonate (method A and A′) (*9*) or directly with the use of dicyclohexylcarbodiimide as the condensing agent (method B) (*10*).

*Method A. DiAc-*L*-TyrGlyOCH$_2$Ph*—The mixed anhydride prepared at -5° from DiAc-L-Tyr (0.01 mole) (*11*), *iso*-butyl chlorocarbonate (0.01 mole), and triethylamine (0.01 mole) in tetrahydrofuran (20 ml.), without isolation, was added with a chilled mixture of GlyOCH$_2$Ph *p*-toluenesulfonate (0.01 mole) (*12*, *13*), triethlylamine (0.01 mole), and chloroform (20 ml.). The reaction mixture was left overnight and crystals appeared after washing the mixture with water, 4 per cent $NaHCO_3$ solution, 2 per cent HCl, and water. The product was recrystallized from ethanol-petroleum ether. Yield, 49 per cent; m.p., 169–170°; $[\alpha]_D^{20}$ $-2.2°$ (c=2, in DMF).

$C_{22}H_{24}O_6N_2$ (412.4): Calcd. N 6.8
Found N 6.8

*Method A′. AcGly-*L*-TyrOCH$_2$Ph*—The mixed anhydride prepared at -5° from AcGly (0.05 mole), isobutyl chlorocarbonate (0.05 mole), and triethylamine (0.05 mole) in DMF (40 ml.) and tetrahydrofuran (50 ml.), without isolation, was added with a chilled mixture of L-TyrOCH$_2$Ph *p*-toluenesulfonate (0.05 mole) (*12 14*), triethylamine (0.05 mole), and chloroform (100 ml.). The reaction mixture was left overnight and washed with water, 4 per cent $NaHCO_3$ solution, 2 per cent HCl solution, and water; the organic layer was dried with exsiccated Na_2SO_4 and concentrated *in vacuo* to leave crystals, which were recrystallized from ethanol-ethyl acetate. Yield, 85 per cent; m.p., 115–117°; $[\alpha]_D^{20}$ $-4.0°$ (c=2, in DMF).

$C_{20}H_{22}O_5N_2$ (370.4): Calcd. N 7.6
Found N 7.7

*AcGly-O-Ac-*L*-Tyr*—The above ester (11.1 g.) was dissolved in *N* NaOH (70 ml.) and left for 30 minutes at room temperature. Acetic anhydride (30 ml.) was added to this solution and it was kept at pH 6 to 8 by adding *N* NaOH. After the mixture was allowed to stand at room temperature for 1 hour, 4 *N* HCl (20 ml.) was added and the product was recrystallized from ethanol-ether. Yield, 7.3 g. (70 per cent); m.p., 183–185°; $[\alpha]_D^{20}$ +11.5° (c=2, in DMF).

$C_{15}H_{18}O_6N_2$ (322.3): Calcd. N 8.7
Found N 8.6

Method B. AcGly-O-Ac-L-TyrGlyOCH$_2$Ph—To a solution of AcGly-O-Ac-L-Tyr (0.01 mole) in tetrahydrofuran (30 ml.), GlyOCH$_2$Ph *p*-toluenesulfonate (0.01 mole), triethylamine (0.05 mole), and N,N′-dicyclohexylcarbodiimide (2.1 g., 0.01 mole) were added in this order. A precipitate of N,N′-dicyclohexylurea formed immediately. After the reaction mixture was allowed to stand overnight, the urea was filtered off, and crystals appeared after the filtrate was washed, by decantation, with water, 4 per cent $NaHCO_3$ solution, 2 per cent HCl, and water. The product was recrystallized from methanol-ether. Yield, 75 per cent; m.p., 163–168°; $[\alpha]_D^{20}$ 0° (c=2, in DMF).

$C_{24}H_{27}O_7N_3$ (469.5): Calcd. C 61.4, H 5.8, N 9.0
Found C 62.6, H 6.7, N 9.6

This compound was contaminated with a trace of N,N′-dicyclohexylurea.

AcGly-L-TyrGly—A solution of the above ester (2.35 g.) in *N* NaOH (15 ml.) and methanol (15 ml.) allowed to stand at room temperature for 1 hour, 2 *N* H_2SO_4 (7.5 ml.) was added, and the mixture was concentrated *in vacuo*. The residue was extracted twice with acetone (total, 60 ml.) and the solution was evaporated *in vacuo*. After it was left to stand overnight in a refrigerator, crystals that appeared were recrystallized from acentone. Yield, 1.0 g. (60 per cent); m.p., 185–186°; $[\alpha]_D^{20}$ 0° (c=2, in DMF).

$C_{15}H_{19}O_6N_3$ (337.3): Calcd. C 53.4, H 5.7, N 12.5
Found C 53.6, H 5.7, N 12.7

AcGly-L-TryAm—A solution of AcGly-L-TyrOCH$_2$Ph (1.85 g.) in methanol (30 ml.), previously saturated with dry NH_3 at 0°, was allowed to stand at room temperature for 2 days. The solution was concentrated *in vacuo* to dryness and the residue was recrystallized from hot 30 per cent ethanol. Yield, 1.0 g. (72 per cent); m.p., 198–199°; $[\alpha]_D^{20}$ −6.5° (c=2, in DMF).

$C_{13}H_{17}O_4N_3$ (279.3): Calcd. C 55.9, H 6.1, N 15.1
Found C 56.0, H 6.1, N 15.3

GlyGlyOCH$_2$Ph p-toluenesulfonate—A mixture of glycine anhydride (20.5 g.), *p*-toluenesulfonic acid monohydrate (40 g.), benzyl alcohol (150 ml.), and benzene (140 ml.) was heated at 110–120° for 4 hours, and the liberated water was removed azeotropically (12, 13). When water no longer distilled off, the reaction mixture was allowed to cool to room temperature and petroleum ether (150 ml.) was added to it. After being left to stand for about 2 hours at 4°, the crystals were collected washed with ether, and recrystallized from ethanol-ether. Yield, 57 g. (80 per cent); m.p.,152–154°.

$C_{18}H_{22}O_6N_2S$ (394.4): Calcd. C 54.8, H 5.6, N 7.1
Found C 54.8, H 5.6, N 7.0

This compound was also prepared from GlyGly by the same procedure.

AcGly-L-TyrGlyGly—AcGly-O-Ac-L-TyrGlyGlyOCH$_2$Ph was prepared from the above ester and AcGly-O-Ac-L-Tyr according to method B. Yield, 80 per cent; m.p., 174–179°; $[\alpha]_D^{20}$ −3° (c=2, in DMF).

$C_{26}H_{30}O_8N_4$ (526.5): Calcd. N 10.6
Found N 10.6

Saponification of the benzyl ester with alkali gave the desired product. Yied, 76 per cent; m.p., 204–207°; $[\alpha]_D^{20}$ 0° (c=2, in DMF).

$C_{17}H_{22}O_7N_4$ (394.4): Calcd. C 51.8, H 5.6, N 14.2
Found C 51.7, H 5.7, N 14.1

DiAc-L-TyrGlyOEt—This compound was prepared from DiAc-L-Tyr and GlyOEt HCl by the method A. Yield, 29 per cent; m.p., 168–170°; $[\alpha]_D^{20}$ 0° (c=2, in DMF).

$C_{17}H_{22}O_6N_2$ (350.4): Calcd. N 8.0
Found N 8.0

N-Ac-L-LyrGly—This compound was prepared from DiAc-L-TyrGlyOCH$_2$Ph (or the above ester) in the same manner as for AcGly-L-TryGly and recrystallized from 50 per cent ethanol. Yield, 71 per cent; m.p., 127–129°; $[\alpha]_D^{20}$ +0.5° (c=2, in DMF).

$C_{13}H_{16}O_5N_2 \cdot H_2O$ (298.3): Calcd. C 52.3, H 6.1, N 9.4
Found C 52.4, H 6.2, N 9.1

N-Ac-L-TyrGlyGly—DiAc-L-TyrGlyGlyOCH$_2$Ph was prepared according to method A. Yield, 68 per cent; m.p., 162–164; $[\alpha]_D^{20}$ 0° (c=2, DMF).

$C_{24}H_{27}O_7N_3$ (469.5): Calcd. N 9.0
Found N 8.9

Saponification gave the desired product. Yield, 77 per cent; m.p., 188–191°; $[\alpha]_D^{20}$ + 1.0° (c=2, in ethanol).

$C_{15}H_{19}O_6N_3$ (337.3): Calcd. C 53.4, H 5.7, N 12.5
Found C 53.1, H 5.8, N 12.3

N-Form-L-TyrGlyGly—N-Form-O-Ac-L-TyrGlyGlyOCH$_2$Ph was prepared from N-Form-O-Ac-L-Tyr (*15*) and GlyGlyOCH$_2$Ph according to method B. Yield, 70 per cent; m.p., 143–146°; $[\alpha]_D^{20}$ +4.8° (c=2, in DMF).

$C_{23}H_{25}O_7N_3$ (455.5): Calcd. N 9.2
Found N 9.2

Saponification gave the desired product which was recrystallized from hot acetone. Yield, 52 per cent; m.p., 111–113°; $[\alpha]_D^{20}$ +32.5° (c=2, in ethanol).

$C_{14}H_{17}O_6N_3 \cdot H_2O$ (341.3): Calcd. C 49.3, H 5.6, N 12.3
Found C 49.3, H 5.6, N 12.5

BzGly-L-Tyr—BzGly-L-TyrOEt was prepared from BzGly and L-TyrOEt HCl according to method A′ or B. The oily ester was treated with NaOH and the resulting BzGly-L-Tyr was extracted with ethyl acetate. The extract was concentrated *in vacuo* and petroleum ether was added to the residue. The product was recrystallized from methanol-ether. Total yield, 20 per cent; m.p., 200–201°; $[\alpha]_D^{20}$ +21.5° (c=2, in DMF).

$C_{18}H_{18}O_5N_2$ (342.3): Calcd. N 8.2
Found N 8.1

BzGly-L-TyrGlyGly—BzGly-L-TyrGlyGlyOCH$_2$Ph was prepared from BzGly-L-Tyr and GlyGlyOCH$_2$Ph according to method B. Yield, 73 per cent; m.p., 190–195°; $[\alpha]_D^{20}$ − 5.0° (c=2, in DMF).

$C_{29}H_{30}O_7N_4$ (546.6): Calcd. N 10.3
Found N 10.3

Saponification gave the desired product which was recrystallized from 80 per cent ethanol. Yield, 70 per cent; m.p., 152–160°; $[\alpha]_D^{20}$ 0° (c=2, in DMF).

$C_{22}H_{24}O_7N_4$ (456.4): Calcd. N 12.3
Found N 12.1

N-Bz-O-Ac-L-Tyr—To a chilled solution of L-TyrOEt HCl (0.01 mole) and triethylamine (0.02 mole) in chloroform (40 ml.), benzoyl chloride (0.01 mole) was added. After being left to stand for 6 hours at room temperature, the mixture was washed with water, 4 per cent $NaHCO_3$ solution, 2 per cent HCl solution, and water; and the organic layer was dried with exsiccated Na_2SO_4 and concentrated *in vacuo* to yield Bz-L-Tyr OEt. This was recrystallized from ethyl acetate-petroleum ether. Yield, 71 per cent; m.p., 119–120°. (This compound has been obtained by another method (*16*); m.p., 120–121° corr.). Saponification of the ethyl ester gave Bz-L-Tyr. Yield, 86 per cent; m.p., 146°. (Also reported elsewhere (*16*); m.p., 164°) Acetylation of Bz-L-Tyr gave the desired product. Yield, 80 per cent; m.p., 155–156°; $[\alpha]_{\mathrm{D}}^{20}$ −78.3° (c=2, in DMF).

$C_{18}H_{17}O_5N$ (327.3): Calcd. N 4.3
Found N 4.2

Bz-L-TyrGlyGly—N-Bz-O-Ac-L-TyrGlyGlyOCH$_2$Ph was prepared from the above substance and GlyGlyOCH$_2$Ph according to method B. Yield, 75 per cent; m.p., 165–170°; $[\alpha]_{\mathrm{D}}^{20}$ −0.5° (c=2, in DMF).

$C_{29}H_{29}O_7N_3$ (531.6): Calcd. N 7.9
Found N 8.1

Saponification of the benzyl ester gave the desired product. Yield, 50 per cent; m.p., 189–191°; $[\alpha]_{\mathrm{D}}^{20}$ +4.0° (c=2, in ethanol).

$C_{20}H_{21}O_6N_3$ (399.4): Calcd. C 60.1, H 5.3, N 10.5
Found C 60.3, H 5.4, N 10.6

This compound could not be obtained in crystalline form from DiBz-L-TyrGlyGlyOCH$_2$Ph.

DiBz-L-TyrGlyOCH$_2$Ph—This compound was prepared from DiBz-L-Tyr* (*17*) and GlyOCH$_2$Ph according to method A′, and recrystallized from DMF-ether. Yield, 64 per cent; m.p., 174–176°; $[\alpha]_{\mathrm{D}}^{20}$ 0° (c=2, in DMF).

$C_{32}H_{28}O_6N_2$ (536.6): Calcd. N 5.2
Found N 5.2

DiBz-L-TyrGlyOEt—This compound was prepared from DiBz-L-Tyr and GlyOEt HCl in the same manner as above. Yield, 68 per cent; m.p., 175–180°; $[\alpha]_{\mathrm{D}}^{20}$ 0° (c=2, in DMF).

$C_{27}H_{26}O_6N_2$ (474.5): Calcd. N 5.9
Found N 5.9

N-Bz-L-TyrGly—This compound was prepared by the saponification of the above two esters. Yield, 73–80 per cent; m.p., 186–187°; $[\alpha]_{\mathrm{D}}^{20}$ 0° (c=2, in DMF).

$C_{18}H_{18}O_5N_2$ (342.3): Calcd. C 63.2, H 5.3, N 8.2
Found C 63.4, H 5·4, N 8.0

N-Cbz-L-TyrGlyGly—N-Cbz-O-Ac-L-TyrGlyGlyOCH$_2$Ph was prepared from N-Cbz-O-Ac-L-Tyr (*18*) and GlyGlyOCH$_2$Ph according to method A. Yield, 60 per cent; m.p., 122–124°; $[\alpha]_{\mathrm{D}}^{20}$ −13.0° (c=2, in DMF).

$C_{30}H_{31}O_8N_3$ (561.6): Calcd. N 7.5
Found N 7.4

Saponification of the benzyl ester gave the desired product. Yield, 78 per cent; m.p., 212–3° (reported elsewhere (*19*); m.p., 213–215°; $[\alpha]_{\mathrm{D}}^{20}$ −21.0° (c=2, in DMF).

---

* This substance was obtained in high yield by maintaining the pH of benzoylation mixture between 7 and 8.

$C_{21}H_{23}O_7N_3$ (429.4): Calcd. N 9.8
Found N 9.6

CbzGly-L-TyrGlyGlyOCH$_2$Ph—This compound was prepared from CbzGly-L-Tyr and GlyGlyOCH$_2$Ph according to method B. Yield, 70 per cent; m.p., 172–176°; $[\alpha]_D^{20}$ 0° (c=2, in DMF).

$C_{30}H_{32}O_8N_4$ (576.6): Calcd. N 9.7
Found N 9.9

Gly-L-TyrGlyGly—The above compound (0.001 mole) was suspended in a mixture of methanol (3 ml.), acetic acid (5.6 ml.), and water (1.4 ml.), and the mixture was treated with hydrogen in the presence of palladium black. The filtrate from the catalyst was repeatedly evaporated *in vacuo* with addition of water. The resulting crystals were recrystallized from water-ethanol. Yield, 71 per cent; m.p., 243° (decomp.); $[\alpha]_D^{15}$ +18.3° (c=2, in water).

$C_{15}H_{20}O_6N_4$ (352.3): Calcd. C 51.1, H 5.7, N 15.9
Found C 51.3, H 5.9, N 16.0

CbzGly-O-Ac-L-Tyr—This compound was prepared by saponification and acetylation of CbzGly-L-TyrOEt (see "AcGly-O-Ac-L-Tyr"). It was recrystallized from ethyl acetate-petroleum ether. Yield, 87 per cent; m.p., 112–114°; $[\alpha]_D^{20}$ +14.5° (c=2, in DMF).

$C_{21}H_{22}O_7N_2$ (414.4): Calcd. N 6.8
Found N 6.6

CbzGly-O-Ac-L-TyrGlyGlyOCH$_2$Ph—This compound was prepared from the above substance and GlyGlyOCH$_2$Ph according to method B. Yield, 75 per cent; m.p., 140–148°; $[\alpha]_D^{20}$ −4.0° (c=2, DMF).

$C_{32}H_{34}O_7N_4$ (618.6): Calcd. N 9.1
Found N 9.4

Cbz-Gly-L-TyrGlyGly—Saponification of the above ester or of CbzGly-L-TyrGlyGlyO-CH$_2$Ph gave the desired product. Yield, 27–31 per cent; m.p., 175–177°; $[\alpha]_D^{20}$ −3.5° (c=2, in DMF).

$C_{23}H_{26}O_8N_4$ (486.5): Calcd. C 56.8, H 5.4, N 11.5
Found C 55.6, H 5.5, N 11.4

Gly-L-TyrGly—CbzGly-L-TyrGlyOCH$_2$Ph was obtained from CbzGly-L-Tyr and GlyO-CH$_2$Ph by method B, but not in a completely pure state. Upon hydrogenolysis, it gave a crystalline product. Total yield, 30 per cent; m.p., 250–254° (decom.); $[\alpha]_D^{15}$ +28.8° (c=1.6, in water).

$C_{13}H_{17}O_5N_3$ (295.3): Calcd. N 14.2
Found N 14.3

This substance was synthesized previously through acid chloride (*19*).

RESULT

pH-Activity Curve—Since no experiments on the optimum pH for α-chymotrypsin and acyl-L-tyrosylglycylglycine have been reported, the pH-activity curve for the enzyme and AcGly-L-TyrGlyGly is given as a representative example. The results are shown in Fig. 1, the optimum pH of the reaction appearing to be 7.7±0.1. Preliminary measurement of the pH dependence of AcGly-L-TyrAm and Ac-L-TyrAm (*20*) in the chymotryptic hydrolysis indicated a maximum at pH 7.9±0.1, the pH-activity curves being similar for both substrates.

For the purpose of comparing susceptibility of all substrates to α-cymotrypsin, a phosphate buffer (0.1 *M*) at pH 7.8 was used as the solvent.

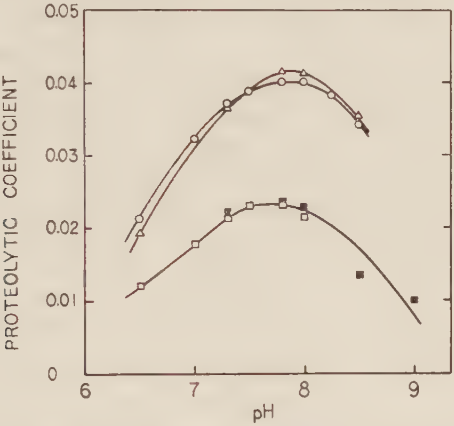


Fig. 1. The pH dependence of the hydrolysis of AcGly-L-TyrGlyGly (□, ■), AcGly-L-TyrAm (○), and Ac-L-TyrAm (△) by chymotrypsin at 30°. The substrate concentration was 0.01 *M*. □, ○, △: 0.1 *M* phosphate buffer. ■: 0.1 *M* with respect to the amine component of trishydroxymethylaminomethane buffer.

Proteolytic Coefficient and Kinetic Constant of the Substrate—In previous reports (*22–23*), the rates of hydrolysis of acyl-L-tyrosinamides, except acetyl derivatives, were measured in methanol-water mixture because of their insolubility in aqueous salt solution and complicating effect of methanol (24).

Table II

Proteolytic Coefficients of Acyl-L-tyrosine Derivatives at Initial Substrate Concentration of 0.01 M

pH 7.8 (0.1 *M* phosphate buffer); temperature, 30°.

Substrate	C[a]	Substrate	C[a]
Bz-L-TyrGlyGly[b]	0.12—0.13	Bz-L-TyrGly	0.0030
CbzGly-L-TyrGlyGly	0.066	Gly-LTyrGly	0.0025
Gly-L-TyrGlyGly	0.034	AcGly-L-TyrGly	0.0021
Cbz-L-TryGlyGly	0.030	Ac-L-TyrGly	0.0020
AcGly-L-TyrGlyGly	0.028		
Form-L-TyrGlyGly	0.023		
BzGly-L-TyrGlyGly	0.02	AcGly-L-TyrAm	0.0435
Ac-L-TyrGlyGly	0.010	Ac-L-TyrAm	0.0438

a) Proteolytic coefficient: C=K/mg. protein N/ml. test solution, where K=(1/minute) log [100/(100-per cent hydrolysis)].

b) Since decreasing values of C were observed during the hydrolysis, the values given are extrapolated initial constants.

Substrates of the acyl-L-tyrosylglycine and acyl-L-tyrosylglycylglycine type, however, are soluble in aqueous salt solution without methanol (when the pH is above 7.0) and they were tested at an initial concentration of 0.01 *M* in aqueous buffer. It was observed that the rate of hydrolysis of the substrates, except benzoyl derivatives, could be approximated by first-order reaction equations, at least over the major portion of the curves. However, in the hydrolysis of substrates Bz-L-TyrGlyGly and BzGly-L-TyGlyGly, the values of the proteolytic coefficient (*1*, *4*) decreased progressively. In this case, an extrapolated initial value (*25*) was taken for comparison of the susceptibility of the substrates. A proteolytic coefficients determined are summarized in Table II.

TABLE III

Proteolytic Coefficients and Kinetic Constants of Acetyl Substance

pH 7.8 (0.1 *M* phosphate buffer): temperature, 30°.

Substrate	Proteolytic coefficient, C				$C_{max.}$[a]	K_m (*M*)	k_3[b]
	0.05 *M*	0.025 *M*	0.01 *M*	0.005 *M*			
AcGly-LTyrGlyGly	0.0190	0.0235	0.0280	0.0330	0.034	0.0621	0.00484
AcGly-L-TyrAm	0.0110	0.0232	0.0435	—	0.217	0.00277	0.00138
Ac-L-TyrAm[c]	—	0.0410	0.0438	0.0466	0.048	0.153	0.0168

a) Maximum proteolytic coefficient: $C_{max.} = k_3/(2.3\,k_m)$

b) In *M*/liter/minute/mg. protein N/ml.

c) Detailed kinetics of the α-chymotrypsin-catalyzed hydrolysis of this substrate at 25° had been reported by Niemann *et al.* (*20*, *26*).

TABLE IV

Inhibitory Activity of Compounds

Substrate (AcGly-L-TyrAm) and inhibitor concentration: 0.01 *M*

pH 7.8 (0.1 *M* phosphate buffer); temperature, 30°.

Inhibitor	C or C_i	Inhibition[a] per cent
None	0.0433	—
Bz-L-Tyr	0.0366	12.4
BzGly-L-Tyr	0.0310	28.4
Phenylpropionic acid	0.0292	32.6
CbzGly-L-Tyr	0.0255	41.1

a) Expressed as per cent change in the proteolytic coefficient (at constant initial substrate concentration) caused by the addition of an inhibitor.

In the hydrolysis of the acetyl derivatives by the enzyme, the values of proteolytic coefficient were determined at various substrate concentrations, and K_m, k_3, and $C_{max.}$ were calculated by the plotting method of Lineweaver and Burk (*1*). Results of these calculations are shown in Table III. Acetyl derivatives were chosen as representative examples because of their ready solubility and simple synthesis.

Inhibition—For the purposes of comparing the inhibitive activity of various compounds tested by the method of Kaufman and Neurath (*21*), first-order reaction constans were used and inhibitory activity was expressed simply as the per cent decrease of proteolytic coefficient of the substrate resulting from the addition of the inhibitor, *i.e.*, per cent inhibition $=(C-C_i)/C\times 100$, where C is the proteolytic coefficient without inhibitor added and C_i the proteolytic coefficient with inhibitor added, at the same initial substrate concentration. AcGly-L-TyrAm was used as the representative substrate in this experiment. Results of these measurements are given in Table IV.

DISCUSSION

As shown in Table II, the proteolytic coefficient for Bz-L-TyrGlyGly is higher than that for all the other substrates used here and the proteolytic coefficient for BzGlyGly is low. It appears that the introduction of glycine between the benzoyl and L-tyrosine groups causes a loss of substrate activity. On the contrary, CbzGly-L-TyrGlyGly is more susceptible to hydrolysis by chymotrypsin than Cbz-L-TyrGlyGly and the same is true between AcGly-L-TyrGlyGly and Ac-L-TyrGlyGly. In addition to the above, since the values of proteolytic coefficients of AcGly-L-TyrAm and Ac-L-TyrAm, and also of AcGly-L-TyrGly and Ac-L-TyrGly, are close and the effect of glycine moiety seems to differ from one case to another. However in the three representative examples of acetyl derivatives shown in Table III, the proteolytic coefficients for AcGly-L-TyrAm and Ac-L-TyrAm change with their concentration at a different rate; consequently, the values of K_m, k_3, and $C_{max.}$ are different. Changes in temperature also seem to cause a large variation in the values of kinetic constants. Although the value of $C_{max.}$ for Ac-L-TyrAm at 30° corresponds to the previously reported value at 25° (*24*, *27*), the values of K_m and k_3 were quite different from those previously reported (*20*, *24*, *26*, *27*).

Although low, the value of proteolytic coefficient for Bz-L-TyrGly, among the acyl-L-tyrosylglycines, was higher than those for other substrates. As was also reported by Bergmann and Fruton, similarly low values were obtained for Cbz-L-TyrGly (*22*) and Cbz-L-PheGly (*23*). When glycylglycine was substituted for glycine in acyl-L-tyrosylglycines, susceptibility of the substrate to hydrolysis by α-chymotrypsin was increased considerably. In the case of acetyl derivatives, amide substrates showed higher proteolytic coefficient than glycylglycine substrates. From the above discussion, it appears, as was also stated by Neurath and Schwert (*4*), that the presence of a negative charge near the susceptible bond causes the loss of substrate activity. On the other hand, a comparison of the values for acyl-L-tyrosinamides and aminoacy-L-tyrosinamides (*1*, *3*) does not indicate whether or not the presence of a positive charge causes stimulation of substrate activity.

From a few number of data presented in Table IV, it appears that the three substances which are products of the enzymatic reaction possess inhibitory activity, but there seems to be no relation between the proteolytic coefficient of a given substrate and inhibitory activity of the corresponding product.

SUMMARY

1. A number of acyl-L-tyrosylglycines and acyl-L-tyrosylglycylglycines were synthesized and tested as substrates for α-chymotrypsin.

2. Using AcGly-L-TyrGlyGly and AcGly-L-TyrAm as substrates, the pH optimum of chymotryptic hydrolysis in phosphate buffer was found to be near 7.8.

3. When proteolytic coefficient (C) at an initial substrate concentration of 0.01 *M* was used as a measure for susceptibility of a substrate to hydrolysis by α-chmotrypsin, the effect of acyl groups on the susceptibility was in a decreasing order of Bz-, CbzGly-, AcGly-, Cbz-, Form-, BzGly- and Ac-, and that for other groups which combined with carboxyl groups of tyrosine residues was in a decreasing order of -Am, -GlyGly, and -Gly.

4. The values of K_m, k_3 and $C_{max.}$ were obtained for several acetyl substances.

5. The inhibitory activities of structural analogues of several substrates for α-chymotrypsin were estimated against AcGly-L-TyrAm.

The author is grateful to Prof. S. Shibuya and Assoc. Prof. Izumiya for their guidance and encouragement, to Dr. M. Winitz for his interest in this study and to Mr. W. Himel for reading the manuscript.

REFERENCES

(*1*) Izumiya, N., and Yamashita, T., *J. Biochem.*, **46**, 19 (1959)
(*2*) Izumiya, N., and Yamashita, T., *J. Biochem.*, **46**, 337 (1959)
(*3*) Yamashita, T., and Izumiya, N., *J. Biochem.*, **46**, 991 (1959)
(*4*) Neurath, H., and Schwert, G. W., *Chem. Rev.*, **46**, 69 (1950)
(*5*) Rosen, H., *Arch. Biochem. Biophys.*, **67**, 10 (1957)
(*6*) Schwartz, T. B., and Engel, F. L., *J. Biochem.*, **184**, 197 (1950)
(*7*) Izumiya, N., and Uchio, H., *J. Biochem.*, **46**, 235 (1959)
(*8*) Yamashita, T., Uchio, H., Izumiya, N., *J. Chem. Soc. Japan*, **80**, 767 (1959)
(*9*) Vaughan, J. R. Jr., and Osato, R. L., *J. Am. Chem. Soc.*, **73**, 5553 (1951)
(*10*) Sheehan, J. C., Goodman, M., and Hess, G. P., *J. Am. Chem. Soc.* **78**, 1367 (1957)
(*11*) Sealock, R. R., *J. Biol. Chem.*, **166**, 1 (1946)
(*12*) Izumiya, N., and Makisumi, S., *J. Chem. Soc. Japan.* **78**, 662 (1957)
(*13*) Zervas, L., Winitz, M., and Greenstein, J. P., *J. Org. Chem.*, **22**, 1515 (1957)

(*14*) Izumiya, N., Uchio, H., and Yamashita, T., *J. Chem. Soc. Japan*, **79**, 420 (1958)

(*15*) Waley, S. G., and Waston, J., *Biochem. J.*, **57**, 529 (1954)

(*16*) Bergmann, M., Ulpts, R., and Camacho, F., *Chem. Ber.* **55**, 2796 (1922)

(*17*) Schultze, A., *Z. physiol. Chem.*, **29**, 467 (1900)

(*18*) Bergmann, M., Zervas, L., Salzmann, L., and Schleich, H., *Z. physiol. Chem.*, **224**, 17 (1934)

(*19*) Abderhalden, E., Abderhalden, R., Weidle, H., Baertich, E., and Morneweg, W., *Fermentforschung*, **16**, 98 (1938)

(*20*) Thomas, D. W., MacAllister, R. V., add Niemann, C., *J. Am. Chem. Soc.*, **73**, 1548 (1951)

(*21*) Kaufman, S., and Neurath, H., *J. Biol. Chem.*, **181**, 623 (1949); *Arch. Biochem.*, **21**, 245 (1949)

(*22*) Bergmann, M., and Fruton, J. S., *J. Biol. Chem.*, **118**, 405 (1937)

(*23*) Fruton, J. S., and Bergmann, M., *J. Biol. Chem.*, **145**, 253 (1942)

(*24*) Kaufman, S., and Neurath, H., *J. Biol. Chem.*, **180**, 181 (1947)

(*25*) Kimmel, J. R., and Smith, E. L., *J. Biol. Chem.*, **207**, 515 (1954)

(*26*) Bernhard, R. A., and Niemann, C., *J. Am. Chem. Soc.*, **79**, 4085 (1957)

(*27*) Schwert, G. W., and Kaufman, S., *J. Biol. Chem.*, **180**, 517 (1949)

The Journal of Biochemistry, Vol. 48, No. 5, 1960

ENZYMATIC REDUCTION OF PICRIC ACID*

By MICHIO TSUKAMURA

(From the Obuso National Sanatorium, Obu, Chita, Aichi-ken)

(Received for publication, March 21, 1960)

There are a few papers concerning the reduction of picric acid occurring as a detoxication process (*1*, *2*). However, there are almost no knowledge on the enzymatic reduction of picric acid. The author (*3*) reported the enzymatic reduction of picric acid by mycobacteria and described several factors influencing the reduction. It is the purpose of the present paper to deal with the reduction of picric acid more systematically.

MATERIALS AND METHODS

Enzyme Materials—Whole cells of *Mycobacterium avium*, strain Jucho, and cell-free preparations prepared from it were used throuhhout. The organism was subcultured in modified Sauton medium, pH 7.0, in which sodium glutamate was substituted for asparagine, for 5 days and washed three times with saline. The cells were pre-incubated before use in saline for 3 hours and then used as enzyme preparations. Cell-free enzyme preparations were prepared as follows. Washed cells were ground with 2 to 3 volumes of aluminium oxide for 60 minutes in a container placed in a freezing mixture. The paste was ten extracted with 0.1 *M* K_2HPO_4 at 0° for 24 hours. A yellowish supernatant was obtained by centrifugation at 3,000 r.p.m. and this was re-centrifuged at 12,000 r.p.m. for 30 minutes. The resulting yellowish supernatant was used as cell-free preparations.

Reaction System—If otherwise described, the following reaction systems were used:

Whole cells: Cell suspension, 1.0 ml.; 0.066 *M* phosphate buffer (KH_2PO_4–Na_2HPO_4), pH 7.8, 1.0 ml.; 0.4 per cent picric acid, 1.0 ml.; 0.1 *M* hydrogen donor, 1.0 ml.; Distilled water or inhibitor, 1.0 ml. (Total: 5.0 ml.). All hydrogen donors were added as sodium salt.

Cell-free preparations: Cell-free preparation, 2.0 ml.; 0.066 *M* phosphate buffer, pH 7.8, 1.0 ml.; 0.4 per cent picric acid, 0.5 ml.; 0.1 *M* hydrogen donor, 0.5 ml.; distilled water or inhibitor, 1.0 ml. (Total: 5.0 ml.). Appropriate control systems were also used.

All the reaction systems were incubated at 37° under static and aerobic conditions.

Determination of Amounts of Reduced Picric Acid—Yellow picric acid is converted into red picramic acid by enzymatic reduction and the amount of picramic acid can be followed quantitatively by colorimetric measurements. The formazan can be easily dissolved in acetone, which, by precipitating bacterial cells, leaves a clear supernatant ready for colorimetric measurement.

To the reaction system, in which whole cells were used as enzyme preparation, was added one volume of acetone (5.0 ml.) and filtered through filter paper. Its optical density was determined by a Leitz's Rouy-Photometer (filter 550 mμ). To the reaction system, in which cell-free preparations were used as enzyme preparations, were added 4 volumes of acetone and the resulting precipitate was filtered through filter paper. Optical density of the latter system was sometimes measured without acetone treatment.

---

* Summary of this paper was reported in *Medicine and Biology* (in Japanese), **33**, 59–62, 270–273 (1954) as preliminary reports.

As will be described later, picramic acid showed its maximal absorption at 490 mμ. However, determination was made at 550 mμ to make indifferent data from optical density of co-existing picric acid. The enzyme activity may be expressed in the term of μg. of dye reduced in given hours by one mg. of cells or protein.

Preparation of Flavoprotein—Flavoprotein was extracted from the organism according to the precedure of Brodie (*4*) with some modifications. Cell-free preparations were heated at 55° for 10 minutes and precipitate of the 0.2 to 0.6 saturation with ammonium sulfate was used for experiments. This flavoprotein preparation could oxidize *ca.* 1 mg. of reduced diphosphopyridine nucleotide (DPNH) per minute per mg. of protein. The amount of protein was measured according to the procedure of Greenberg (*5*). Supernatant obtained by trichloroacetic acid treatment of this preparation showed a flavin-typical absorption spectrum (260 mμ, 380 mμ and 460 mμ).

RESULTS AND DISCUSSION

I. Reduction of Picric Acid by Whole Cells—1. Identification of the red formazan: Intensity of red color varied with the amount of cells. It was accelerated markedly by shaking reaction system ih a water bath (at 37°).

A supernatant of a reaction system, which changed into red, was evaporated under reduced pressure. The resulting concentrate was placed onto Toyo filter paper (No. 50) and developed by ascending paper chromatography (Table I). The red formazan showed only one spot and gave

TABLE I

Paperchromatography

(Ascending system. Toyo filter paper, No. 50)

Solvent	Sample	Red Spot	Yellow Spot
n-Butanol Saturated with 3% NH_4OH	Test Solution	0.08	0.80
	Picramic Acid	0.06	—
	Picric Acid	—	0.80
n-Butanol Saturated with 0.1 *M* K_2HPO_4	Test Solution	0.07	0.66
	Picramic Acid	0.07	—
	Picric Acid	—	0.65
Amylacetate Saturated with 3% NH_4OH	Test Solution	0.05	0.24
	Picramic Acid	0.05	—
	Picric Acid	—	0.24

Standard picramic acid was prepared by reducing picric acid by sodium hydroxide and sodium sulfide or by sodium hydroxide and glucose.

Rfs similar to those of picramic acid in different solvent systems. Furthermore, the red spots were cut off and extracted with $M/60$ phosphate buffer, pH 7.8. The extracts were determined for their absorption spectra by a Hitachi's spectrophotometer. They showed one maximum absorption at 490 mμ, which is characteristic for picramic acid. Picric acid showed maxi-mum absorption at 430 mμ (Fig. 1). In addition, it was shown that the

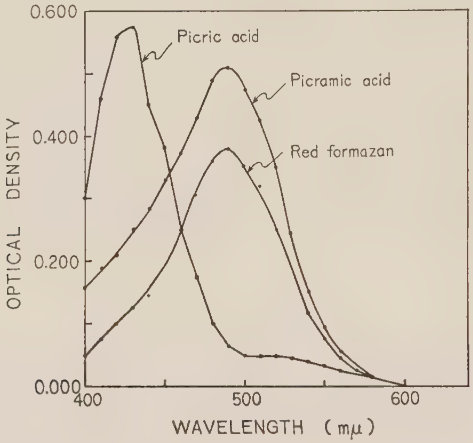


FIG. 1. Absorption curves of picric acid, picramic acid (standard) and red formazan.
(The red formazan was eluted into $M/60$ phosphate buffer, pH 7.8, from the red spot obtained by the paper chromatography using *n*-butanol saturated with $1\,N$ NH_4OH as a solvent.)

red formazan is soluble in water, acetone and alcohol and insoluble in ether, chloroform, amylacetate and *n*-butanol. This solubility also agreed with that of standard picramic acid. The red formazan was identified with picramic acid. The reaction may be described as follows:

$$\text{Picric Acid} + 6H = \text{Picramic Acid} + 2H_2O$$

2. Straight line relatioship between the amount of reduced picric acid and optical density: There was a linear correlation between the amount of reduced picric acid or picramic acid and optical density. The amount of reduced picric acid is equimolar with the amount of picramic acid produced.

3. Straight line relationship between the amount of enzyme (cells) and the amount of reduced picric acid: This correlation was also demonstrated at least in the range between 2 to 32 mg., wet weight, of cells per ml.

4. Heat inactivation: Influence of heating was examined in a reaction system containing 10 mg. of cells per ml. and $0.02\,M$ lactate in final concentrations. The activity of reduction of picric acid was reduced to 75 per cent, 30 per cent and 0 per cent after heating for 10 minutes in a

water bath at 50°, 60° and 70°, respectively. It was also demonstrated that the activity disappears after acetone extraction of cells.

5. Curves of reduction of picric acid: Curves of reduction of picric acid varied with the amount of cells and with the hydrogen donor (Fig. 2). When the amount of cells was relatively large, the reduction occurred first rapidly and thereafter slowly (Fig. 2). When it was relatively small, the reduction increased linearly untill more than 24 hours. It appeared that, at least, during the first several hours there exists a liner correlation between the amount of reduced picric acid and the incubation time. If the reaction system is shaken in a water bath, a significant red change occured within one hour.

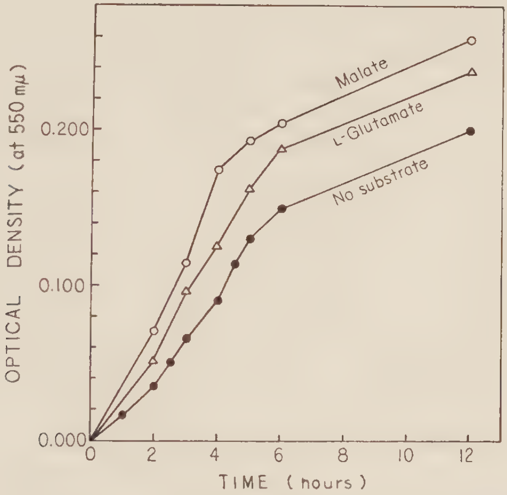


Fig. 2. Curves of reduction of picric acid in the presence of different hydrogen donors (Final concentration: Cells, 16 mg./ml.; Hydrogen donor, 0.02 *M*)

6. Comparison of reduction of picric acid under aerobic and anaerobic conditions: Thunberg's tubes were prepared attached to the Leitz's Rouy-Photometer and curves of the reduction were observed both under aerobic and anaerobic (2 mmHg.) conditions. The reduction occurred rather more rapidly under the aerobic condition.

7. Reduction of picric acid in the presence of different hydrogen donors: The effect of hydrogen donors on the reduction varied from donor to donor. In the test organism, addition of glucose and malate most markedly increased the reduction. Pyruvate, acetate, succinate, lactate, formate, fructose, D-xylose, glycerol, nitrate, ammonium chloride, glutamate, alanine, asparagine, etc., at a some extent increased the reduction. Citrate, glycine, nitrite, fumarate, and benzoate had no effect or rather decreased the reduction.

The reduction occurred more markedly by increasing the donor concentration.

Influences of hydrogen donors on the reduction varied from strain to strain. This can be seen in Table II. The fact may mean a difference in metabolic pattern among the strains.

TABLE II

Ratios of Reduction of Picric Acid in the Presence of Different Hydrogen Donors Added in a Final Concentration of 0.02 M

Hydrogen Donor (Sodium Salt)	*Mycobacterium avium*, Jucho	*Mycobacterium tuberculosis*, H37Rv	*Escherichia coli*, Denken
No Donor	1.00	1.00	1.00
Glucose	1.99	1.01	3.97
Glycerol	1.20	1.00	/
Lactate	1.15	1.15	6.50
Pyruvate	1.40	1.17	6.60
Malate	1.31	1.22	1.00
Acetate	1.12	1.09	0.89
Succinate	1.12	0.95	1.00
L-Glutamate	1.24	/	/
Citrate	1.00	1.00	/

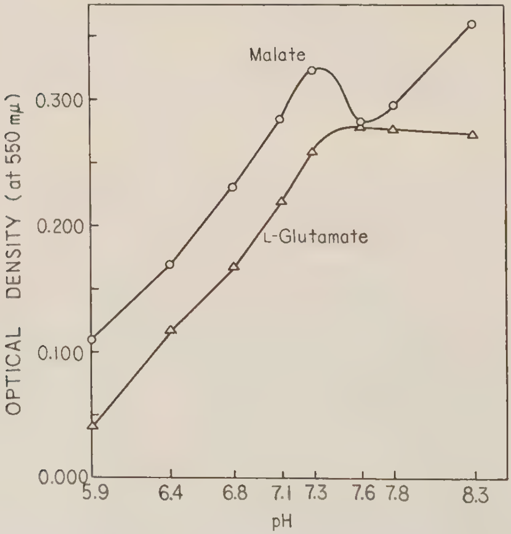


FIG. 3. Effect of pH on the reduction of picric acid (Final concentrations: Cells, 30 mg./ml.; Hydrogen donor, 0.02 *M*, 12 hours)

8. Effect of pH on the reduction of picric acid: The reduction was affected by the pH of the reaction system. There was a tendency that

rate of reduction increased with incresing the pH (lowering the hydrogen ion concentration) (Fig. 3).

9. Michaelis constant: The reduction of picric acid was measured at various concentrations of picric acid in a system without hydrogen donor. The Michaelis constant was calculated as $2.1 \times 10^{-3} M$.

10. Effects of inhibitors on the reduction of picric acid: Heavy metal ions, $HgCl_2$, $AgNO_3$ and $CuSO_4$, exhibited the most significant inhibitory effect and other inhibitors had no significant inhibitory effect. KCN acted rather accelerative. The results suggest that flavoprotein may take a part in the reduction (Table III).

TABLE III

Effects of Inhibitors on the Reduction of Picric Acid by Whole Cells
(Cells, 10 mg./ml. Determined after 16 hours in the absence of substrate)

Inhibitor	Final Concentration	Rate of Inhibition
KCN[1]	$2\times10^{-3}M$	0%
Urethane	2×10^{-3}	0
Sodium azide	2×10^{-3}	13
NaF	2×10^{-3}	0
Sodium arseniate	2×10^{-3}	0
Malonic Acid	2×10^{-3}	10
Monoiodoacetic Acid	2×10^{-3}	10
Octyl Alcohol	2×10^{-3}	20
Sodium Benzoate	2×10^{-3}	0
2, 4-Dinitrophenol	2×10^{-3}	20
$HgCl_2$	2×10^{-3}	100
$HgCl_2$	2×10^{-4}	100
$AgNO_3$	2×10^{-3}	100
$AgNO_3$	2×10^{-4}	100
$CuSO_4$	2×10^{-3}	100
$CuSO_4$	2×10^{-4}	96
$NaIO_3$	2×10^{-3}	75
p-Nitrophenol	2×10^{-3}	50

Rate of inhibition $=(1-E/E_0)\times100\%$.

1) Since coexistence of picric acid and KCN produced a red color, inhibition rate was calculated reducing the optical density due to this color.

11. Differentiation from enzyme system reducing p-nitrobenzoic acid (PNBA): Yamashina (*6*) stated that the enzyme system reducing PNBA in *Bacillus pumilus* is inhibited by nitrite. However, the reduction of picric acid is not inhibited by nitrite. In addition, it was demonstrated that the

reduction of picric acid by *Mycobacterium avim* is inhibited by sulfathiazole, whereas the reduction of PNBA by the organism is not inhibited by sulfathiazole (*7*, *8*).

Formation of PABA from PNBA occurred in the presence of sulfathiazole in concentrations of 125 to 4,000 μg./ml., which was demonstrated by separating spots of sulfathiazole and PABA in ascending paper chromatography using *n*-butanol saturated with 1 *N* acetic acid as solvent (R_f of sulfathiazole: 0.08 to 0.10; R_f of PABA: 0.80). Michaelis constant for the reduction of picric acid was $2.1 \times 10^{-3} M$ and that for the reduction of PNBA was $6.1 \times 10^{-4} M$ (*9*). Enzyme system responsible for the reduction of PNBA appears to differ from that for the reduction of picric acid. The enzyme system responsible for the reduction of picric acid appears to be different from that responsible for the reduction of PNBA.

II. Reduction of Picric Acid by Cell-Free Preparations—1. Reduction cuaves and effects of various hydrogen donors on the reduction of picric acid: Reduction of picric acid by cell-free preparations occurred more rapidly (Fig. 4). Effects of hy hydrogen donors on the reduction by cell-free preparations differed from those by whole cells. Unlike the case of whole cells, glucose and malate did not increase the reduction. On the contrary, L-glutamate and lactate accelerated the reduction markedly. This fact suggests that enzyme systems responsible for the metabolisms of glucose and malate may exist in relatively large particles or cell membrane and those for the metabolisms of glutamate and lactate may exist in relatively

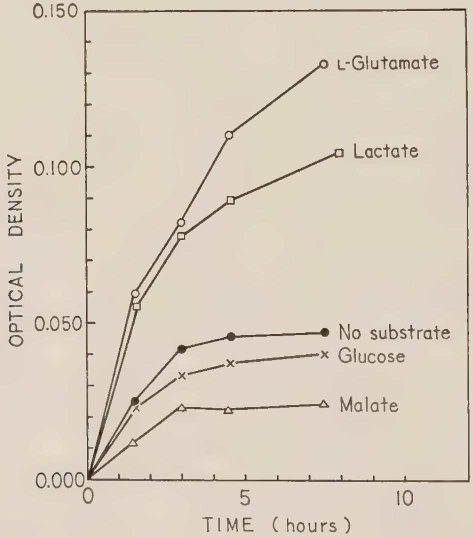


FIG. 4. Reduction of picric acid by cell-free preparations in the presence of different substrate (Final concentration of substrate: 0.01 *M*)

small particles. Each mg. of protein of an enzyme preparation reduced 150 μg. of picric acid per hour in the presence of 0.01 M L-glutamate.

2. Michaelis constant: The Michaelis constant was $2.0 \times 10^{-3} M$ for the reduction of picric acid by cell-free preparations.

3. Effects of inhibitors on the reduction of picric acid: The results are shown in Table IV. $HgCl_2$ and $AgNO_3$ exhibited the most significant inhibitory effect and octanol followed them. KCN accelerated significantly the reduction.

TABLE IV

Effects of Inhibitors on the Picric Acid Reduction by Cell-Free Preparations[1)]

Inhibitor	Final Concentration	Rate of Inhibition
KCN	$2 \times 10^{-3} M$	−100%
Urethan	2×10^{-3}	0
Arseniate	2×10^{-3}	0
Malonate	2×10^{-3}	0
NaF	2×10^{-3}	9
Azide	2×10^{-3}	55
Monoiodoacetate	2×10^{-3}	55
Benzoate	2×10^{-3}	20
Octyl Alcohol	2×10^{-3}	60
$HgCl_2$	2×10^{-4}	100
$AgNO_3$	2×10^{-4}	100
$CuSO_4$	2×10^{-4}	25
$NaNO_2$	2×10^{-3}	0
p-Aminobenzoic Acid	2×10^{-3}	0

1) Reaction system consisted of 5.0 ml. of the reaction mixture, each ml. of which can reduce 37 μg. of picric acid per hour in the presence of 0.01 M sodium L-glutamate. The amount of reduced picric acid was determined after 6 hours of incubation.

4. Effect of dilution on the reduction of picric acid: Unlike the case of whole cells, the activity of cell-free preparations decreased by the dilution progressively.

III. Mechanism of the Enzymatic Reduction of Picric Acid—The results of the effects of inhibitors on the reduction suggested that flavoprotein may concern the reduction. It was examined thus whether a system consisting of flavoprotein and DPNH can reduce picric acid or not.

An example of results is shown in Table V. The rest DPNH just before adding picric acid was calculated as 60 μg./ml. It appears, however, that DPNH undergoes oxidation after addition of picric acid. Therefore, if it is supposed that the rest DPNH be completely oxidized, the amount of DPNH expected to give the reduction of picric acid is considered to be

900 μg. Molar ratio between the amount of DPNH, which disappeared in the presence of flavoprotein, and that of reduced picric acid was calculated as 4:1. This ratio appears considerably to resemble the theoretical ratio.

TABLE V

Reduction of Picric Acid by DPNH-Flavoprotein System

Reaction System (Total: 3.0 ml.)	Final Concentration		
	I	II	III
Flavoprotein in *M*/15 Phosphate Buffer, pH 7.3	0.3 mg. of protein/ml.		0.3 mg. of protein/ml.
DPNH[1]	460 μg./ml.	460 μg./ml.	
Amount of DPNH after 18 min.[1] (25°C)	60 μg./ml.	300 μg./ml.	
Total Amount of DPNH Oxidized by Flavoprotein	720 μg.	0 μg.	
Added with 0.5 ml. of 0.4 per cent picric acid			
Optical Density at 550 mμ after 10 min.	0.310	0.138[2]	0.111
Total Amount of Picric Acid Reduced by Flavoprotein	80.5 μg.[3]		

1) Determined by optical density at 340 mμ. DPNH was prepared according to the procedure of Umbreit *et al.* (*10*). DPN used was a 95 per cent pure product of the National Biochemicals Corporation (U.S.A.).

2) Reduction due to the residual sodium hydrosulfite used for the reduction of DPN.

3) Calculated from the difference of optical densities between the systems I and II.

In view of the results, picric acid is believed to accept hydrogen directly or indirectly from flavoprotein and to be changed to picramic acid.

It was observed previously by the author that sulfateiazole and homosulfamine inhibit the reduction of picric acid by *Mycobacterium avium*. It was also observed that sulfathiazole inhibits the oxidation of DPNH by flavoprotein, whereas homosulfamine does not inhibit it (*7*, *11*). This finding suggests that, since function of flavoprotein remains intact by homosulfamine and, irrespective of this fact, the reduction of picric acid is inhibited by the presence of homosulfamine, picric acid may accept hydrogen through some factors, which exist between flavoprotein and cytochrome systems.

SUMMARY

Picric acid can be reduced enzymatically into picramic acid. It is conceivable that picric acid accepts hydrogen through flavotprotein.

Yellow picric acid can be converted into red picramic acid by whole cells and cell-free preparations under aerobic conditions. Picric acid appears

to be utilizable as a redox dye.

The author wishes to express his appreciation to Dr. R. Katsunuma, director of the Obuso National Sanatorium, Prof. S. Hibino, Nagoya University, and Prof. N. Katsunuma, Osaka University, for their kind encouragement and advice. The author is also indebted to Mr. S. Mizuno for his technical assistance.)

REFERENCES

(*1*) Stekol, J. A., *Ann. Rev. Biochem.*, **10**, 265 (1941)
(*2*) Handler, P., and Perlzweig, W. A., *Ann. Rev. Biochem.*, **14**, 617 (1945)
(*3*) Tsukamura, M., *Kekkaku* **25**, 655 (1950)
(*4*) Brodie, A. F., *J. Biol. Chem.*, **199**, 835 (1952)
(*5*) Greenberg, D. M., *J. Biol. Chem.*, **82**, 545 (1929)
(*6*) Yamashina, I., *Bull. Chem. Soc. Japan*, **27**, 85 (1954)
(*7*) Tsukamura, M., *Chemotherapy*, **4**, 43 (1956)
(*8*) Tsukamura, M., *Medicine & Biology*, **34**, 37 (1955)
(*9*) Tsukamura, M., *Kekkaku*, **29**, 388 (1954)
(*10*) Umbreit, W. W., Burris, R. H., and Stauffer, J. F., "Monometric Techniques and Tissue Metabolism," Burgess Publ., Minneapolis, (1949)
(*11*) Tsukamura, M., *Chemotherapy*, **2**, 176 (1954)

The Journal of Biochemistry, Vol. 48, No. 5, 1960

PHOSPHORUS METABOLISM IN HUMAN ERYTHROCYTE

IV. DESTRUCTION OF ADENINE NUCLEOTIDES IN STORED BLOOD*

By MAKOTO NAKAO**, MASAMITI TATIBANA
AND HARUHISA YOSHIKAWA

*(From the Department of Physiological Chemistry and Nutrition,
Faculty of Medicine, University of Tokyo, Tokyo)*

(Received for publication, April 6, 1960)

By general consent, 3 weeks have been considered to be the maximum permissible time of storage, when whole blood is preserved in acid-citrate-dextrose medium at 4°. Beyond this time, the physiological viability decreases rapidly and at the end of several week storage the erythrocytes lose the ability to survive when transfused into a recipient. Gabrio *et al.* (*1*) attributed the phenomenon of erythrocyte deterioration to internal metabolic processes, among which most important was the lowering of ATP level in the cells. They explained that a rather high level of ATP, as reflected by the high value of the Michaelis constant, is required in the hexokinase reaction, wherein glucose is phosphorylated by ATP to yield G-6-P and utilized. This opinion is supported by further observations that adenosine or inosine when added to 4 weeks old blood produces repletion of ATP and improves the post-transfusional viability of the cells (*2*, *3*). If blood is stored more than several weeks, however, the ATP level is not increased any longer by addition of inosine unless adenine is supplemented simultaneously (*4*). This must be attributed to depletion of the adenine moiety which is needed for the resynthesis of ATP inside the cells since only adenine is effective among purines and pyrimidines so far examined (*5*).

To elucidate the mechanism of the destruction of ATP during storage, it appeared worth while to pursue the qualitative and quantitative changes of the phosphorus compounds in the erythrocytes. Although there have been several investigations along this line, most of them have been done by less reliable methods for separating phosphate esters (*6–8*). Prankerd

---

* This work was aided by a grant from the Scientific Research Fund of the Ministry of Education. The principal part of this work has appeared already in *J. Japan. Biochem. Soc.*, **31**, 526 and 679 (1959).

Abbreviation as follows: AMP, adenosine 5′-monophosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; Attp, adenosine tetraphosphate; IMP, inosine 5′-monophosphate; DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; PGA, phosphoglyceric acid; DPGA, diphosphoglyceric acid; SMP, sugar monophosphate; SDP, sugar diphosphate; IP, inorganic orthophosphate.

** Present address: Department of Biochemistry, Faculty of Medicine, Gumma University, Maebashi.

(*9*) employed paper chromatography but this method is not satisfactory for quantitative analysis of the compounds as pointed out in a previous paper (*10*). Ion exchange resin chromatography has been proved to give more accurate analytical values when used to examine the quantitative picture of the phosphorus compounds in blood (*11*, *12*). Chromatograms of nucleotides in fresh and stored blood obtained by Potter and Takagi are illustrated in a review by Gabrio *et al.* (*13*) without detailed description. The present communication deals with the phosphate changes during storage of blood which have been observed by the use of the column chromatography on cation exchange resin and the radioactive isotope of phosphorus.

MATERIALS AND METHODS

Materials—Acid-citrate-dextrose (ACD) medium was made according to the Formula B, Ministry of Public Welfare (1.33 g. of glucose, 1.32 g. of sodium citrate, and 0.48 g. of citric acid in 100 ml.), and, to prepare acid-citrate-dextrose-inosine (ACDI) medium, inosine was added in a final concentration of 0.025 *M*.

Each collecting bottle which contained 50 ml. of either ACD or ACDI medium received 200 ml. of blood from donors of blood-type A, and the contents of the five bottles with ACD and of those with ACDI medium were mixed, respectively. The pooled ACD blood and ACDI blood thus prepared were devided into 200 ml. and 100 ml. aliquots. All the samples were kept at 37° for 1 hour as usual and then preserved in an ice-box of 4°. When radioactive phosphorus was added, 1 ml. of P^{32} in isotonic NaCl containing 0.04 mg. carrier P/ml. as sodium phosphate buffered at pH 7.4 was introduced into 100 ml. of ACD or ACDI blood. The amount of P^{32} added per 100 ml. was generally about 250 μc. All the manipulations were performed aseptically.

Analytical Methods—To 1 volume of the erythrocytes which were washed three times with ice-cold physiological saline and freed from leucocytes was added 2 volumes of ice-cold 0.6 *N* perchloric acid (PCA), mixed and after 5 minutes centrifuged at 5,000 r.p.m. for 15 minutes. The precipitate was extracted twice with 0.3 *N* PCA and the extracts were combined. In some experiments the re-extraction of the precipitate was omitted to avoid increase of the volume of the extract. In the latter case, the efficiency of the single extraction was estimated previously from the per cent recovery of radioactive phosphate added to ice-cold blood cells immediately before the extraction and a correction was made for the value in analysis.

The PCA extract was neutralized with KOH using phenol red as the indicator and the precipitate of $KClO_4$ was centrifuged off after chilling in a freezing mixture. The supernatant was lyophilized, the residue was dissolved in 20 ml. of water, chilled and the precipitate was removed again by centrifugation. The supernatant solution was diluted with water to 500 ml. and submitted to chromatography on Dowex-1, X10, formate (mesh 200–400) column of $0.8\,cm^2 \times 15$ cm. with gradient elution technique developed by Hurlbert *et al.* (*14*). The fractions of 10 ml. volume were collected and their optical density at 260 mμ. and, if necessary, at 275 mμ. and the radioactivity of P^{32} were determined as described in a previous paper (*12*). The radioactivity was corrected for decay.

Further resolution and identification of the compounds in the eluates were achieved as described in previous papers (*5*, *12*, *15*). IMP was separated by the aid of Norit A and by rechromatography on Dowex-1 chloride column (*16*) and sugar phosphates were by column chromatography employing borate according to Khym and Cohn (*17*).

Specific radioactivity of labile P of ATP and ADP was determined as follows: one milliliter of blood sample was deproteinized with 6 ml. of 0 6 *N* PCA in the cold, centrifuged, and to 5 ml. of the supernatant was added a small amount of Norit SX 30, which had been treated with octanol according to Threlfall (*18*). The charcoal was spun down and washed three times with cold water containing a small amount of carrier P when radioactivity was to be assayed. Then, the charcoal was half-dried by soaking water with filter paper, and heated with 2 ml. of 1 *N* HCl at 100° for 7 minutes to hydrolyze the adsorbed nucleotides. After centrifugation, 1 ml. of the supernatant was neutralized with NaOH, 1 ml. each of 2 *N* sulfuric acid and 2.5 per cent ammonium molybdate were added, and extracted with 2 ml. of isobutanol. A half milliliter of the butanol extract was evaporated on a sample dish and counted under a Geiger-Müller tube. To determine the quantity of the liberated phosphate the charcoal which adsorbed the nucleotides was washed with water without addition of carrier P, and the compounds were hydrolyzed in the same way. The inorganic phosphate in the hydrolyzate was determined by Fiske-Subbarow's method (*19*).

Hypoxanthine was determined enzymatically with xanthine oxidase (*20*) and uric acid colorimetrically according to Allport and Keyea (*21*). Other assay methods were the same as in previous papers (*5*, *12*).

The activity of 5′-nucleotidase was determined by measuring the liberated inorganic phosphate (*22*) from AMP in the presence of magnesium ion and arsenate.

RESULTS

Acid Soluble Phosphorus Compounds in Stored Blood—To a sample of ACD blood was added radioactive orthophosphate and, after keeping the blood at 37° for an hour, the blood was preserved for 4 weeks at 4°. At the end of the period, erythrocytes were separated and analyzed for acid-soluble phosphorus compounds by ion exchange resin chromatography. The results are presented in Fig. 1.

A negligible amount of ultraviolet absorbing substance or radioactive compound passed through the column unabsorbed. By subsequent running water through the column, a considerable amount of hypoxanthine was eluted out. It was identified by the characteristic absorption spectrum, the maximum of which shifted from 248 to 252 mμ. on adjustment of the solution from acid to alkaline pH. Elution by formate system gave peaks with ultraviolet absorption corresponding to AMP, ADP, ATP, Attp-like substance (*12*), IMP, DPN fraction and TPN fraction. These nucleotides were identified by absorption on active charcoal, rechromatography and determination of absorption spectra. It is noteworthy that both AMP and IMP were present in considerably high concentration in stored blood cells while in fresh cells they were scanty. Xanthine and uric acid were also eluted out overlapping winh DPN fraction.

SMP and SDP fractions appeared as radioactive peaks. 2,3-DPGA which is the most prominent non-nucleotide phosphate ester in fresh erythrocyte was markedly decreased. Rechromatography of SDP fraction on Dowex-1, X10, formate column (Fig. 2) showed two major overlapping P^{32} peaks, the smaller preceding the larger. Ester of the second peak was alkali labile, most of the radioactivity being liberated as orthophosphate after keeping its solution in 0.025 N NaOH at 100° for 30 minutes (370 c.p.m.

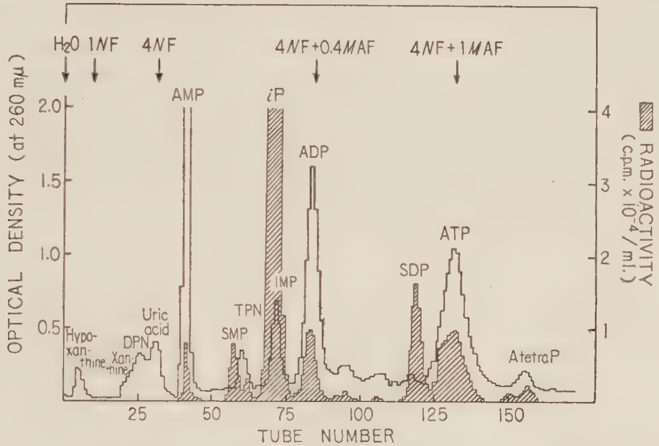


FIG. 1. Chromatography of PCA extract of erythrocytes which had been preserved in ACD medium for 4 weeks with addition of P^{32}. The volume of the mixing flask was 400 ml. The content of the reservoir was changed at the arrows. F: formic acid; AF: ammonium formate. Optical density at 260 mμ was represented by empty column and radioactivity by shaded column.

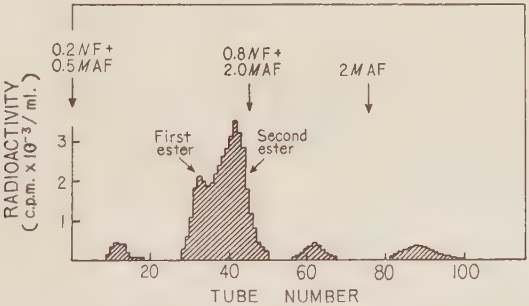


FIG. 2. Dowex-1 formate rechromatography of the sugar diphosphates in human blood stored for 4 weeks. Five eluent fractions containing sugar diphosphates from a Dowex-1 formate were pooled and concentrated in vacuo over KOH. The concentrate was neutralized to pH 8.0 with 5 N KOH and the organic phosphates were precipitated by addition of a few drops of 20 per cent barium acetate and 4 volumes of alcohol. The precipitate was treated with Amberlite 120 (H^+) and was put on a 0.8 cm^2. × 10 cm. Dowex-1, X10, formate column. Rechromatography was performed by gradient elution by the use of 400 ml. mixer and the eluting solution was changed at the arrows. Ten milliliter fractions were collected.

out of total 440 c.p.m.). Far less portion of P^{32} was liberated from the ester of the first peak (78 c.p.m. out of total 525 c.p.m.). The two esters behaved differently in paper chromatography. The second ester, larger in amount, had a higher R_f value and coincided with the authentic sample of F-1,6-DP. The first ester which was smaller in amount corresponded to the ester constituted the larger part of the hexose diphosphate mixture obtained from fresh blood. Color reactions for sugar (*23*) were examined on both of the esters, the results beibg summarized in Table I. These results and the assay values of phosphorus indicate that the first ester appears to be glucose-1,6-diphosphate, the existence of which in red blood cells was demonstrated recently by Bartlett (*11*).

TABLE I

Color Reactions of the Two Phosphate Esters in Hexose Diphosphate Fractions

The samples were obtained from the froactions shown in Fig. 2.

Reagents	The first ester (G-1,6-DP)	The secon ester (F-1,6-DP)
Orcinol	negative	negative
Resorcinol	negative	red
Cysteine-carbazole	negative	pink to purple
Diphenylamine	negative	blue
Cysteine-sulfuric acid	yellow; changing to greenish yellow on standing	not tested

Phosphate Changes during Storage in the Cold—Fresh ACD and ACDI blood specimens were stored in a refrigerator maintained at 4° after preincubation at 37° with added P^{32}. Table II summarizes the values of the phosphate esters which were obtained by ion exchange chromatographic analyses of the stored blood cells.

1. When the specimens were transferred in the refrigerator the temperature of the blood declined to reach 4° within 24 hours. It may be seen from the Table that inorganic phosphate accumulated markedly and 2,3-diphosphoglycerate was broken down, while the concentration of ATP, ADP and AttP-like compound underwent little change, if any.

2. At the end of 4 week preservation 2,3-diphosphoglycerate of ACD blood nearly disappeared and concurrently inorganic phosphate accumulated enormously in the cells. At this stage the level of ATP also declined to less than one fourth of the original. On the contrary, AMP and IMP were increased several times. The concentration of ADP remained unchanged up to this time. When the ACD blood which had been stored for 4 weeks was incubated with added inosine, ATP was restored to some extent, ADP decreased markedly and AMP almost diappeared. The increase of IMP was also conspicuous. It was observed that an abrupt rise of 2,3-DPGA and a fall of inorganic phosphate occurred during the incubation with added inosine. The changes observed in the concentration of 2,3-

DPGA, SMP, SDP and inorganic phosphate suggests that the addition of inosine stimulates the rate of phosphorylation inside the stored blood cells.

Table II

Changes of Phosphorus Compounds of ACD and ACDI Blood during Storage in the Cold

Conditions of the storage are described in the text. All values are expressed as μmoles per 100 ml. blood corrected for dilution with the media and loss by deproteinization.

Duration of storage	1 h.1) at 37°	24 h.	4 w.			8 w.	
Medium	ACD	ACD	ACD	ACDI	ACD2)	ACD2)	ACDI2)
DPN fr	1.3	1.3	1.8	2.4	—	—	—
TPN fr.	1.4	1.6	1.6	1.1	—	—	—
AMP	2.2	2.5	8.1	6.9	0.6	trace	trace
ADP	7.6	5.9	7.6	5.2	2.7	trace	trace
ATP	41	43	9.3	8·7	16.5	2.2	7
Attp-like substance	2.1	1.8	0.7	0.9	—	0.4	0.7
IMP	1.1	1.5	4.6	14.6	27	6.9	22
IP	54	96	470	354	31	—	65
SMP fr.	8.7	10.3	8.5	16.2	82	12.5	103
SDP fr.3)	5.9	7.7	8.0	11.0	27	7.5	16
2,3-DPGA	188	132	3.2	13.8	124	2.4	109

1) Preincubation for the storage of blood.

2) At the end of the preservation period, the specimen was incubated at 37° for 2 hours with supplimentation of the followings: 4 week ACD blood, 0.005 *M* inosine; 8 week ACDI blood, 0.025 *M* inosine; 8 week ACD blood, none.

3) Values are expressed as P.

The results obtained from the blood sample which had been preserved supplemented with a rather small amount of inosine, ACDI blood, were not much different from those of ACD blood as seen in the Table, but 2,3-DPGA still remained undepleted and SDP and SMP fractions were increased during the preservation. IMP was also increased appreciably. However, these differences observed between ACD and ACDI blood samples were less than the changes evoked by incubation of aged ACD blood with addition of inosine.

3. At the end of 8 week storage, both of the ACD and ACDI bloods were incubated for 2 hours at 37°, the latter being supplemented with an additional quantity of inosine. As already reported in the previous papers (*4*, *5*), adenine nucleotides as well as 2,3-DPGA almost disappeared whereas sugar phosphates were increased to some extent during the storage of ACD

blood. When the aged blood was incubated with supplementation of inosine, 2,3-DPGA and sugar phosphates increased markedly, but the level of ATP remained still very low. This indicates that even in this stage the blood cells respond to added inosine with synthesis of phosphate esters without an appreciable elevation of ATP concentration. A marked increase of IMP was again observed after incubation with added inosine.

Incorporation of P^{32}—Specific activities of acid-soluble phosphorus compounds in the stored erythrocytes were calculated as summarized in the Table III.

TABLE III

Specific Activity of Phosphorus Compounds in Preserved Erythrocytes

Blood specimens are the same as given in Table II. Values are expressed as c.p.m./μg. P. Figures in parentheses are the specific activities of labile phosphorus in ADP and ATP which have been calculated under assumption that the stable phosphorus of the adenine nucleotides has the same specific activity as the phosphorus of AMP and there is a rapid equilibriation between the labile phosphate groups of ATP owing to the presence of active adenylate kinase (*24*).

Duration of storage	1 h. at 37°	24 h.	4 w.	
Medium	ACD	ACD	ACD	ACDI
AMP	680	870	900	1230
ADP (labile P)	2660 (4660)	3150 (5430)	1610 (2320)	—
ATP (labile P)	3320 (4680)	3910 (5430)	1640 (2010)	2380 (2955)
IMP	—	—	—	2140
IP	5940	4850	1900	1930
SMP fr.	1820	3000	1660	2870
SDP fr.	1400	2420	1670	2070
2,3-DPGA	191	1340	1605	2820

During the preincubation at 37° for 1 hour, incorporation of P^{32} preceeded to a considerable extent resulting in essentially the same pattern as in an experiment reported in the previous paper where heparinized blood was used (*12*) except that the specific activity of 2,3-DPGA was much lower in ACD blood than in heparinized blood. At the end of storage for 24 hours, the specific activity of 2,3-DPGA rose considerably. Labile P of ATP as well as of ADP attained a specific activity comparable to that of cell inorganic P during the preincubation and after 24 hour storage in the cold, and the specific activity of labile P in ADP and ATP grew still further exceeding that of inorganic phosphate. The specific activities of SMP and SDP fractions also attained the highest values at the end of 24 hour storage, and then declined gradually to come into equilibrium with the specific activities of inorganic phosphate and 2,3-DPGA during the course of 4 week storage. The specific activity of inorganic P declined continuously to equilibrium, whereas those of labile P of ADP and ATP were still higher than the equilibrium level although the values decreased to less than one half of the initial.

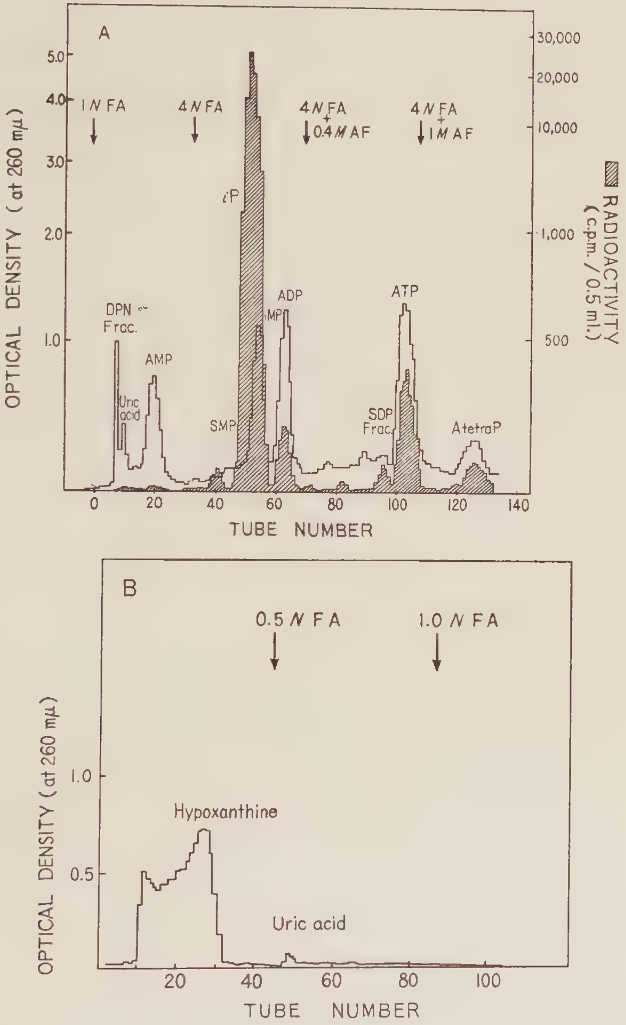


FIG. 3. Chromatography of PCA extract of erythrocytes and plasma which had been separated from preserved ACD blood. To 100 ml. of the blood stored for 4 weeks was added 290 μc. of P^{32} and kept at 4° for additional 40 hours before separation of erythrocytes and plasma. The volume of the mixing flask was 400 ml. Symbols as in Fig. 1. A, blood cells; B, plasma.

In ACDI medium the incorporation of P^{32} into phosphorus compounds inside the cell was greater than in ACD medium and the equilibration of the specific acitivity among the phosphates was not reached yet. It deserved notice that the specific activity of IMP was significantly higher than that of AMP.

Fig. 3 presents the chromatographic patterns of PCA extracts of erythrocytes and plasma separated from stored ACD blood. P^{32} was added at the end of 4 week storage and the blood was kept for additional 40 hours at 4°. It was demonstrated that even long-stored blood cells incorporated P^{32} into ADP and ATP to some extent in the cold but AMP and 2,3-DPGA were apparently unlabeled. Plasma contained an appreciable amount of hypoxanthine which had been diffused out of the cells and a small amount of uric acid. The presence of xanthine was not confirmed by this experiment.

Hypoxanthine and Uric Acid—The concentration of hypoxanthine was very low in fresh blood and increased markedly during the period of preservation. The values determined by the use of xanthine oxidase were 25.2 μmoles per 100 ml. ACD blood at the end of 4 weeks and 49.5, 57.0, 54.4, 50.0 and 50.7 μmoles per 100 ml. ACD blood at the end of 8 weeks.

The concentration of uric acid did not change significantly. After 10 weeks it was 2 mg. per cent, namely within the normal ranga.

5′-Nucleotidase Activity—There have been several communications upon phosphatase which is responsible for dephosphorylation of nucleotides in red blood cells (*25*, *26*). Table IV presents enzymatic liberation of inorganic phosphate from AMP in the presence of hemolysate. Arsenate was added to the assay system to prevent the reutilization of inorganic phosphate for the phosphorolysis of nucleosides in the hemolysate.

Table IV

Liberation of Inorganic Phosphate from Adenosine 5′-Monophosphate

Assay system: 0.5 ml. of buffer, 3 μmoles of AMP, 10 μmoles of magnesium chloride, 0.5 ml. of 1 : 1 fresh blood hemolysate which had been dialysed for 8 hours, and sodium arsenate (final concentration 0.01 *M*). Total volume, 1.2 ml. Incubation, 5 hours at 37°.

Buffer	Liberated inorganic phosphate
1 *M* acetate buffer, pH 4.5	2.7 μg.
0.1 *M* Tris buffer, pH 7.4	7.9 ,,
1 *M* glycine buffer, pH 8.5	1.8 ,,

DISCUSSION

The increase of inorganic phosphate in erythrocytes during storage is roughly equivalent to the decrease of 2,3-DPGA and ATP as seen in Table II. During the period of storage at 4°, the specific activity of intracellular inorganic phosphate decreased continually, probably, due to dilution by steady accumulation of unlabeled inorganic phosphate which originates from 2,3-DPGA in the cell. These facts indicate that most of the inorganic phosphate liberated from esters is retained within the cells against a concentration gradient as pointed out by Gabrio *et al.* (*13*). The red cell membrane seems to transport phosphate inwards by active processes concurrent with very slow outward leakage of the anion by simple diffusion.

The total concentrations of the nucleotides including IMP in blood samples preserved for various periods of time were 54 at the beginning, 30 after 4 weeks and 10 μmoles/100 ml. after 8 weeks of storage, as calculated from the values given in Table II. The levels of hypoxanthine determined enzymatically were 31 for 4 weeks old blood and 61 μmoles/100 ml. for 8 weeks old blood. Since normal fresh human blood containes only 1 to 3 μmoles/100 ml. of hypoxanthine and xanthine (*27*) and no appreciable amount of xanthine was found in the preserved blood samples, one may say that the decrease in total nucleotide during preservation of ACD blood agrees fairly well with the increase in hypoxanthine. The absence of xanthine oxidase in blood cells was also indicated, because its presence would result in a marked increase of uric acid in the stored blood. From these findings and the concentrations of nucleotides during storage indicated in Table II, it may be concluded that ATP in erythrocytes is metabolized through ADP and then AMP to hypoxanthine.

The present authors (*24*) have obtained a highly active preparation of adenylate kinase from human erythrocytes and shown the enzyme activity is not decreased appreciabley during a 6-week storage of the bood. Kashket and Denstedt (*28*) also demonstrated the presence of the enzyme in erythrocytes. The high activity of adenylate kinase in erythrocytes accounts for the fact that the specific activity of labile P of ADP was very close to the mean value of the specific activities of two labile Ps of ATP. The values of the equilibrium constant calculated from the data indicated above were around 1.5 under various conditions of preservation, reasonably close to that for muscle adenylate kinase reported by Kalckar (*29*). When re-phosphorylation of ADP to ATP coupled with glycolytic process inside the cells has been impaired by long term storage, AMP is formed by the action of adenylate kinase upon accumulated ADP, and in turn, undergoes irreversible conversion to hypoxanthine, thus resulting in lowering of the level of the total adenine nucleotide. There are two possible pathways for the breakdown of AMP, one being *via* adenosine and the other *via* IMP. The presence of an adenylate and adenosine deaminase in human erythrocytes has been reported by Conway and Cooke (*30*) and others

(*28*, *31*, *32*) and our observation indicates that 5′-nucleotidase activity is present in the cell.

It is known that human red cells contain an active purine nucleoside phosphorylase which attacks inosine and guanosine, but not adenosine (*33*). By the action of this enzyme activity inosine undergoes phosphorolysis with liberation of hypoxanthine as fast as it is formed, adenine nucleotides thus being converted ultimately into hypoxanthine during the storage of blood. The possibility that hypoxanthine is formed from inosine by the action of nucleosidase may be ruled out by the fact that the conversion does not take place in hemolysate unless inorganic phosphate is added (*31*).

A larger amount of IMP was found in the blood cells stored in ACDI medium than in ACD medium and the specific radioactivity of IMP in stored ACD blood was significantly higher than that of AMP. These findings suggests that the deamination of AMP is not the only way for the formation of IMP, but IMP may be newly synthesized also from hypoxanthine. A mechanism for the synthesis of IMP has been demonstrated by Preiss and Handler (*34*) who showed that in hemolysate phosphoribosyl pyrophosphate is formed from ribose 5′-phosphate and ATP and IMP is produced by the back reaction of pyrophosphorolysis from phosphoribosyl pyrophosphate and hypoxanthine. Regeneration of adenine nucleotides in long-stored blood when adenine and inosine are supplemented can be accounted for by the analogous mechanism (*4*).

Dische (*35*) showed that the ribose 1-phosphate formed from the metabolism of nucleosides in fluoride-treated hemolysate led to the formation of hexose phosphates presumably *via* ribose 5-phosphate. The presence of both phosphoglucomutase and phosphoribomutase in human erythrocytes was reported by Guarino and Sable (*36*). Ribose 5-phosphate probably enters Warburg-Dickens hexose monophosphate shunt and is further metabolized to lactic acid, *via* the Embden-Meyerhof pathway. An abrupt rise in sugar phosphates in stored red blood cells during incubation with added inosine indicates that a considerable portion of the hexose monophosphate shunt mechanism and glycolysis is active. Thus the energy-rich phosphates

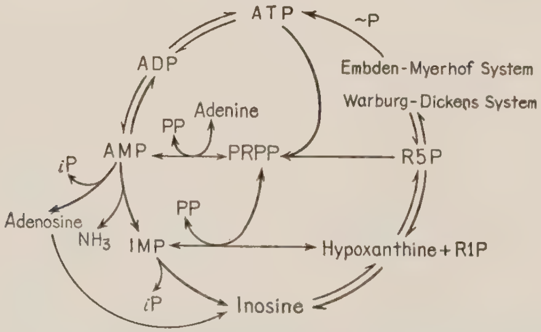


FIG. 4. Nucleotide metabolic pathways in human erythrocyte.

which occur as the intermediates of the Embden-Meyerhof pathway may serve to phosphorylate ADP.

Fig. 4 presents a schematic picture of metabolic pathways of nucleotides in human erythrocyte inferred from our studies and the findings of other workers. The irreversibility of the process may be attributed to the reactions induced by deaminases.

It has been the conclusion of our previous communications (*4*, *5*) that supplementation of inosine alone is not sufficient to restore the declined ATP level in the long-stored erythrocyte but simultaneous addition of adenine with inosine is necessary. Further studies have demonstrated that if blood is stored with added adenine and inosine, not only the level of ATP but also the osmotic resistance and the morphological integrity of the cells are maintained as long as for 8 weeks (*37*).

SUMMARY

Changes in the concentrations of phosphorus compounds in red blood cells during storage in acid-citrate-dextrose medium and in the same medium supplemented with inosine at 4° were persued by column chromatography with the use of P^{32} as the indicator. The most prominent change was decrease of 2,3-diphosphoglyceric acid and concurrent accumulation of inorganic orthophosphate. Adenosine triphosphate content declined continually during the storage due to retrogression of re-phosphorylation process and at the end of 8 weeks the total amount of adenine nucleotide became extremely low. On the other hand, inosine monophosphate was increased considerably, and hypoxanthine accumulated in an amount which was correspondent to the decrease of the total of nucleosides including inosine monophosphate.

After incubation of the stored blood with added inosine, an appreciable rise of sugar phosphates and 2,3-diphosphoglycerate was observed, while the total amount of adenine nucleotides was slightly decreased though adenosine triphosphate was increased markedly. Inosine monophosphate was also increased by incubation with added inosine, and its considerably high specific radioactivity suggests that inosine monosphosphate may be formed not only from adenosine monophosphate by deamination but also synthetized newly from hypoxanthine.

In conclusion, ATP in erythrocyte undergoes gradual destruction to hypoxanthine, the enzyme involved in the process being adenosine triphosphatase, adenylate kinase, adenylic deaminase, 5′-nucleotidase, adenosine deaminase and nucleoside phosphorylase. Supplementation of inosine alone is not sufficient to restore adenosine triphosphate level in the long-stored erythrocytes.

REFERENCES

(*1*) Gabrio, B. W. and Finch, C. A., *J. Clin. Invest.*, **33**, 242 (1953)

(2) Gabrio, B. W., Donohue, D, M., and Finch, C. A., *J. Clin. Invest.*, **34**, 1509 (1955)

(3) Donohue, D. M., Finch, C. A., and Gabrio, B. W., *J. Clin. Invest.*, **35**, 562 (1956)

(4) Nakao, M., Nakao, T., Tatibana, M., Yoshikawa, H., and Abe, T., *Biochem. et Biophys. Acta*, **31**, 564 (1959)

(5) Nakao, M., Nakao, T., Tatibana, M., and Yoshikawa, H., *J. Biochem.*, **47**, 661 (1960)

(6) Rapoport, S., *J. Clin. Invest.*, **26**, 591 (1954)

(7) Pappius, H. M., and Denstedt, O. F., *Can. J. Biochem. Physiol.*, **32**, 293 (1954)

(8) Gabrio, B. W., Hennesey, M., Thomasson, J., and Finch, C. A., *J. Biol. Chem.*, **215**, 357 (1955)

(9) Prankerd, T. A. J., *Biochem. J.*, **64**, 209 (1956)

(10) Yoshikawa, H., Nakao, M., Miyamoto, K., and Yanagisawa, I., *J. Biochem.*, **46**, 83 (1959)

(11) Bartlett, G. R., *J. Biol. Chem.*, **234**, 449 (1959)

(12) Yoshikawa, H., Nakao, M., Miyamoto, K., and Tatibana, M., *J. Biochem.*, **47**, 635 (1960)

(13) Gabrio, B. W., Finch, C. A., and Huennekens, F. M., *Blood*, **11**, 103 (1956)

(14) Hurlbert, R. B., Schmitz, H., Brumm, A. F., and Potter, V. R., *J. Biol. Chem.*, **209**, 23 (1954)

(15) Tatibana, M., Nakao, M., Miyamoto, K., Sekiguchi, T., and Yoshikawa, H., *J. Biochem.*, **46**, 711 (1959)

(16) Cohn, W. E., and Carter, C. E., *J. Am. Chem., Soc.*, **72**, 4273 (1950)

(17) Khym, J. K., and Cohn, W. E., *J. Am. Chem. Soc.*, **75**, 1153 (1953)

(18) Threlfall, C. I., *Biochem. J.*, **65**, 694 (1957)

(19) Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925)

(20) Kalckar, H. M., *J. Biol. Chem.*, **167**, 429 (1947)

(21) Allport, N. L., and Kwyer, J., "Colorimetric Analysis," edited by Chapman and Hall (1957)

(22) Martin, J. B., and Doty, D. M., *Anal. Chem.*, **66**, 375 (1949)

(23) Ashwell, G., in S. P. Colowick and N. O. Kaplan, "Methods in Enzymology," Vol. **III**, Academic Press, New York, p. 73, (1957)

(24) Tatibana, M., Nakao, M., nnd Yoshikawa, H., *J. Biochem.*, **45**, 1037 (1958)

(25) Clarkson, E. M., and Mayzels, M. J., *J. Physiol.*, **116**, 112 (1952)

(26) Tsuboi, K. K., and Hudson, P. B., *Arch. Biochem. Biophys.*, **43**, 399 (1953)

(27) Jorgensen, S., and Poulsen, H. E. L., *J. Am. Pharm. Toxicol.*, **11**, 287 (1955)

(28) Kashket, S., and Denstedt, O. F., *Canad. J. Biochem. Physiol.*, **36**, 1057 (1958)

(29) Kalckar, H. M., *J. Biol. Chem.*, **148**, 127 (1943)

(30) Conway, E. J., and Cooke, R., *Biochem. J.*, **33**, 479 (1939)

(31) Yoshizumi, S., *Igaku Kenkyu* (*Acta Medica*), **29**, 1663 (1959)

(32) Rubinstein, D., and Denstedt, O. F., *Canad. J. Biochem. Physiol.*, **34**, 927 (1956)

(33) Gabrio, B. W., and Huennekens, F. M., *Biochim. et Biophys. Acta*, **18**, 585 (1955)

(34) Preiss, J., and Handler, P., *J. Biol. Chem.*, **225**, 759 (1957)

(35) Dische, Z., in W. D. Elroy and B. Glass, "Phosphorus Metabolism," Vol. **1**, The Johns Hopkins Press, Baltimore, p. 171, (1951)

(36) Guarino, A. J., and Sable, H. Z., *J. Biol. Chem.*, **215**, 515 (1955)

(37) Nakao, M., Nakao, T., Arimatsu, Y., and Yoshikawa, H., *Proc. Japan Acad.*, **36**, 43 (1960)

The Journal of Biochemistry, Vol. 48, No. 5, 1960

PHOSPHORUS METABOLISM IN HUMAN ERYTHROCYTE

V. INCORPORATION OF P^{32} DURING VERY SHORT TIME INTERVALS*

By MASAMITI TATIBANA, KANJI MIYAMOTO, TAKASHI ODAKA
AND MAKOTO NAKAO**

(From the Department of Physiological Chemistry and Nutrition, Faculty of Medicine, and the Institute of Infectious Diseases, University of Tokyo, Tokyo)

(Received for publication, April 19, 1960)

It has been demonstrated by several workers that the uptake of orthophosphate ion by human erythrocytes might not be a process of simple diffusion, but involve glycolytic activity of the cells. However, the experimental results are not in accord with each other in detail. Gourley (*1*) studied the distribution of added orthophosphate-P^{32} among acid-soluble phosphorus compounds in red blood cells and proposed a hypothesis that the phosphate enters the cell, forming adenosine triphosphate (ATP) at the cell surface. On the other hand, Prankerd and Altman (*2*) reached a different conclusion that 2, 3-diphosphoglyceric acid bears a precursor relationship to ATP, being in equilibrium with 1, 3-diphosphoglyceric acid. Schild and Maurer (*3*) showed the presence of creatine phosphate in the human erythrocytes and an active incorporation of P^{32} into this compound as well as into ATP.

It was thought to be necessary, therefore, to re-examine the distribution of added radioactive phosphorus among the acid-soluble phosphorus compounds in erythrocyte by means of more precise methods, employing much shorter time intervals of contact with P^{32}.

METHODS

The methods used in the experiments have been described previously (*4*). Freshly drawn venous blood was heparinized and centrifuged, the white "buffy layer" was removed, and the packed celles were resuspended in the plasma. The same procedure was repeated. The cell suspension was shaken gently in a water bath at 37° for 30 minutes in an atmosphere of air containing 5 per cent carbon dioxide to assure the steady state of cell metabolism. The pH of the blood was constant within the range 7.40 to 7.43 during the incubation period. Radioactive orthophosphoric acid was treated with HCl in a boiling water bath before use, and osmotic pressure, phosphate concentration and pH of the solution were adjusted to be equal to those of blood plasma. The isotope solution was added to the erythrocyte suspension at the end of the preincubation period in an amount of 30 to 80 μc. per ml. At four different time intervals, three being 10 to 90 seconds and one 30 minutes after the addition of P^{32}, aliquots (10 or 30 ml.) of the suspension were pipetted directly into an ice-cold 20 per cent trichloroacetic acid of equal volume. The subsequent operations were carried out in a cold room kept at 2°. As the zero-time control, the preincubated blood cell suspension was extracted with trichloroacetic acid prior to the addition of P^{32}. Alcohol-insoluble barium salt was precipitated from the extract and decationized with the use of Amberlite IR 120(H^+). The solution was neutralized with ammonia and to it was added an amount of KH_2PO_4 solution containing 4 mg. of P. The mixture was diluted to 1 liter with water and passed through a Dowex-1, X2, chloride column, 7 cm.$\times$0.5 cm^2. The column was eluted with 1000 ml. of a solution of $M/40$ ammonium chloride and $M/100$ potassium borate, followed by washing with water. Four milligrams of carrier P was put again into the column and the same eluting solution was passed through. The flow rate was regulated to about 1.5 ml. per minute. By this treatment the excess of radioactive inorganic orthophosphate was removed nearly completely, leaving phosphate esters on the column. The subsequent elution was carried out according to the method of Cohn and Carter (*5*) with minor modifications.

---

* This work was aided by a grant from the Scientific Research Fund of the Ministry of Education. Principal part of this work has been published in *J. Japan. Biochem. Soc.*, **30**, 595 and 667 (1958).

** Present address: Department of Biochemistry, Faculty of Medicine, Gumma University, Maebashi.

In some experiments, perchloric acid was used for deproteinization and the gradient elution with formic acid and ammonium formate deviced by Hurlbert *et al.* (*6*) was employed for the analysis of acid-soluble phosphorus compounds. In this case, removal of large quantity of inorganic P^{32} from the column was effected by the following procedure. When the zone of orthophosphate on the column reached the lower tip of the column, which was readily detected with a radiation survey meter, the elution was interrupted and 300 to 500 ml. of 0.1 M ammonium formate was run.

The eluate fractions of 10 ml. volume were collected in either case and the optical density at 260 mμ. (E_{260}) and the radioactivity were measured. Re-chromatography was conducted as described in the previous paper.

RESULTS AND DISCUSSION

Fig. 1 represents the ion exchange resin chromatogram obtained from the blood after 10 second incubation with added P^{32}. By re-chromatography of the eluents, only ATP and ADP among well defined phosphorus compounds were shown to be labeled with P^{32} to a detectable extent, while the other substances, such as sugar phosphates and phosphoglycerates were not. The same results were obtained by incubation of the blood with P^{32} for short time intervals not more than 90 seconds. After 30 minutes incubation, P^{32} was incorporated into sugar phosphates and phosphoglycerates. In Table I is made a comparison between specific activities of ATP and 2, 3-diphosphoglycerate at 30 minute point. It is clearly seen that the ratio $P^{32}:P$ of ATP is much larger than that of 2, 3-diphosphoglycerate.

The results thus obtained revealed that the incorporation of P^{32} into 2, 3-diphosphoglycerate was rather sluggish one, contrary to the finding obtained by Prankerd and Altman (*2*). The explanation for this discrepancy has been given in the previous paper (*4*).

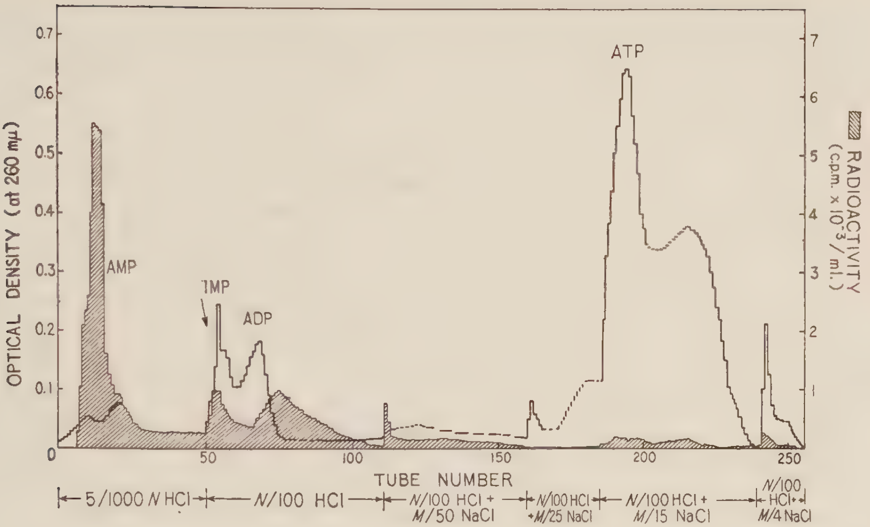


Fig. 1. Dowex-1 chloride chromatography of acid-soluble phosphorus compounds from the blood sample incubated with added P^{32} for 10 seconds. The experimental details are given in the text. The eluting solutions are written under the abscissa. Ultraviolet absorption is represented by empty and radioactivity by cross-hatched areas. Arrows represent the unidentified phosphates which took up P^{32}.

Table I

Specific Activities of 2, 3-Diphosphoglycerate and ATP at the End of 30 Minute Incubation with Added P^{32}

Phosphorus of 2, 3-diphosphoglycerate was assayed by colorimetric determination after digestion. The values for ATP were calculated from the ion exchange chromatograms.

Experiment	Specific Activity (*c.p.m./μg. P*)		Ratio of the Specific Activity
	2, 3-Diphosphoglycerate	ATP	
No. 1	850	7200	1 : 8.5
No. 2	3200	17000	1 : 5.3

To obtain further information about the reaction sequence involved in the uptake of inorganic phosphate into organic phosphates inside the cells, the time course of P^{32}-incorporation into ATP was determined over a short period of incubation time. It is essential for such a study that a steady state is maintained during the incubation. As seen in Table II, this was the case in the Experiment 1, where the variation in the amount of ATP and ADP was negligibly small during the course of incubation. In the Experiment 2, a slight decrease of ATP and a corresponding increase of

ADP were observed at the 40 second and 30 minute points. This is probably due to decomposition of ATP during kept as its barium salt in a wet state for a fairly long time until it was analysed. The P^{32} content of ATP corrected for the accidental fluctuations in the amount of ATP in the erythrocytes are plotted against incubation time in Fig. 2. Although the activity increases linearly with time a short but distinct lag is seen in the rising-up. This suggests that a certain intermediary step may exist between inorganic orthophosphate in plasma and ATP in the cell.

TABLE II

Concentration of ATP and ADP in Blood Incubated for Different Periods of Time after Addition of P^{32}

Values of the concentration are expressed as μmole per 100 ml. blood.

Experiment 1				Experiment 2			
Incubation Time	ATP	ADP	ATP+ADP	Incubation Time	ATP	ADP	ATP+ADP
15 sec.	38.5	5.0	43.5	10 sec.	40.7	5.5	46.2
50 ,,	37.1	5.6	42.7	20 ,,	39.3	5.1	44.4
90 ,,	36.0	5.6	41.6	40 ,,	34.7	11.7	46.4
30 min.	36.0	5.6	41.6	30 min.	29.1	13.8	42.9

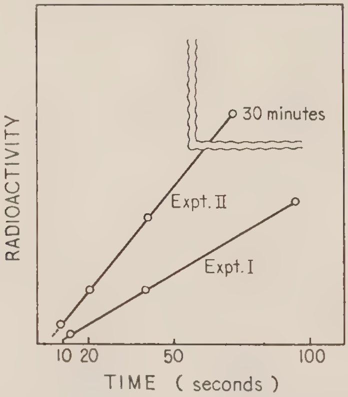


FIG. 2. Time course of the activity of ATP in blood which was incubated with P^{32}. The radioactivity is expressed in arbitrary unit.

It is likely that the entry of phosphate involves phosphorylation carried out at the cell surface in the reaction of phosphoglyceraldehyde oxidation to 1, 3-diphosphoglycerate. A corollary of this assumption is that in short-time experiments the 1, 3-diphosphoglycerate should have a higher radioactivity than ATP. Unfortunately, the 1, 3-diphosyhoglycerate is extremely unstable (7) so that separation and assay of this compound are hard to be accomplished by the present method. Indeed, there appeared highly radioactive unidentified phosphorus compounds at the end of 10 seconds of incubation other than ATP and ADP (Fig. 1), but none of them coincided with 1, 3-diphosphoglycerate in hydrolysability in acid and alkali nor with creatine phosphate in behavior against molybdenolysis. Furthermore, these compounds were often demonstrated in the zero-time controls. Consequently, much biological significance should not be placed upon the presence of these compounds with radioactivity in very early period of incubation though it could not be completely denied that they may be actually involved in the posphorus metabolism in human erythrocyte.

Incorporation of P^{32} into ADP was clearly detected by means of radioautography taken from the paper chromatograms as early as at the end of 10 second incubation. The α-phosphate group of both ATP and ADP possessed no detectable radioactivity up to 90 seconds. In a 40 second experiment the ratio of the specific activity of ADP to that of ATP was calculated from the analytical values obtained from the chromatograph. As given in Table III, the specific activity of ADP is roughly a half that of ATP. This fact suggests that a rapid equilibration among labile phosphate groups of ADP and ATP is attained as soon as P^{32} is incorporated into γ-phosphate group of ATP.

TABLE III

Specific Activities of ATP and ADP after Incubation with Added P^{32} for 40 Seconds

Thirty milliliters of blood, from which leucocytes and platelets had been removed, were incubated with P^{32} (approximately 1 mc.) for 40 seconds, and at the end of the incubation extracted with 2 volumes of ice-cold 0.6 *N* perchloric acid. The extract was concentrated in a froken state to about 20 ml. and the precipitate formed was removed by filtration. The filtrate was diluted to about 500 ml. and passed through Dowex-1 formate, ×8, column, 15 cm.×0.9 cm². with addition of 10 mg. carrier P. The subsequent elution was carried out by a slight modification of the method of Hurlbert *et al.* (*6*).

Compound	Tube Number	E_{260}	Radioactivity *c.p.m./ml.*	$\frac{c.p.m./ml.}{E_{260} \times 10^3}$
ADP	55	0.625	1420	2.34
	56	0.650	1622	2.50
	57	0.500	1094	2.18
			mean	2.34
ATP	120	3.97	15460	3.92
	122	1.42	5860	4.12
	124	0.62	2400	3.88
			mean	3.97

SUMMARY

As an attempt to elucidate the mechanism of phosphate uptake by human erythrocyte, P^{32} incorporation into acid-soluble organic phosphates during very short time period of incubation with added P^{32} orthophosphate was pursued by the use of ion exchange resin chromatography.

The P^{32} incorporation into 2, 3-diphosphoglycerate was found to be a rather sluggish one. In an incubation time up to 90 seconds no detectable labeling of the ester was obtained. Even after 30 minutes of incubation the specific activity of 2, 3-diphosphoglyceric acid was several times as small as that of ATP.

ATP and ADP became labeled to a detectable extent as early as 10 seconds after the addition of P^{32}, when neither sugar phosphates nor phosphoglycerate took up practically no P^{32}. The incorporation of P^{32} into ATP increases with time linearly with a very short lag. This suggests that there is an intermeniary step between inorganic orthophosphate in plasma and ATP in the cell. In addition to ATP, and ADP, several unidentified compounds with very early incorporation of P^{32} were found. However, their biological significance is ambiguous because they were present some times even in zero-time controls.

A very rapid isotopic equilibration among the labile phosphate groups of ATP and ADP was demonstrated.

REFERENCES

(*1*) Gourley, D. R. H., *Arch. Biochem. Biophys.*, **40**, 1 (1952)
(*2*) Prankerd, T. A. J., and Altman, K. I., *Biochem. J.*, **58**, 622 (1954)
(*3*) Schild, K. T., and Maurer, W., *Biochem. Z.*, **323**, 235 (1952)
(*4*) Yoshikawa, H., Nakao, M., Miyamoto, K., and Tatibana, M., *J. Biochem*, **47**, 635 (1960)
(*5*) Cohn, W. E., and Carter, C. E., *I. Am. Chem. Soc.*, **72**, 4273 (1950)
(*6*) Hurlbert, R. B., Schmitz, H., Brumm, A. F., and Potter, V. R., *J. Biol. Chem.*, **209**, 23 (1954)
(*7*) Neglein, E., and Brömel, H., *Biochem. Z.*, **303**, 132 (1938)

The Journal of Biochemistry, Vol. 48, No. 5, 1960

STUDIES ON SULFATASES OF THE LIVER OF CHARONIA LAMPAS

III. DIGESTION OF CELLULOSEPOLYSULFATE

By NORIKO TAKAHASHI

(*From the Department of Chemistry, Faculty of Science,
Nagoya University, Nagoya*)

(Received for publication, April 22, 1960)

Takahashi and Egami considered that the degradation of cellulosepolysulfate by the crude liver extract of *Charonia lampas* proceeds as follows (*1*):

$$\underset{\text{(S-rich)}}{\text{cellulosepolysulfate}} \xrightarrow[\text{cellulosepolysulfatase}]{\qquad\qquad} \underset{\text{(S-poor)}}{\text{cellulosesulfate}}$$

$$\xrightarrow[\text{polysaccharases}]{\qquad\qquad} \underset{\text{and glucose}}{\text{glucosesulfates}} \xrightarrow[\text{glucosulfatase}]{\qquad\qquad} \text{glucose}$$

The present report describes the hydrolysis of cellulosepolysulfate (S-rich) by cellulosepolysulfatase to give cellulosesulfate (S-poor) and the digestion of the latter by polysaccharases to give glucose and glucose monosulfate.

MATERIALS AND METHODS

Preparation of Substrates—Potassium cellulosepolysulfate (S content, 18.0 per cent) and cellulosesulfate (S content 6.8 per cent) were both prepared as usual by sulfation of cellulose powder with chlorosulfonic acid in pyridine. (reaction mixture: 8.4 ml. of chlorosulfonic acid and 3 g. of cellulose powder, for cellulosepolysulfate. 0.36 ml. of chlorosulfonic acid and 1 g. of cellulose powder, for cellulosesulfate.)

Preparation of Enzyme Solution—Cellulosepolysulfatase ("preparation B"), separated from polysaccharases by adsorption with CM-cellulose, and purified polysaccharases were prepared by the methods previously described (*2*). Specific activity of cellulosepolysulfatase in the crude extract and in "preparation B" are 0.2 and 1.2 respectively.

Measurement of Enzyme Activities—Unless otherwise mentioned, the activities were determined with a reaction mixture containing 0.5 ml. enzyme solution, 3 mg. polysaccharide sulfate, adjusted to 1 ml. with 0.1 *M* acetate buffer, pH 6.0. A few drops of chloroform were added and the reaction mixture was incubated at 37°. Inorganic sulfate was determined as previously described (*1*). Total sugar was determined by the anthrone method of Scott and Melvin (*3*), and reducing power was determined by the method of Somogyi (*4*), after deproteinization with zinc hydroxide gel.

Charcoal Adsorption for Analysis of the Reaction Products—For the identification of the products formed on digestion of cellulosepolysulfate, charcoal adsorption method devised by Rapport, Meyer and Linker (*5*) was used with slight modifications: Reaction mixture (about 5 ml.) was boiled and centrifuged. To the supernatant, 400 mg. of Norit pretreated with *N* HCl was added, stirred and centrifuged. The sediments were washed

3 times with 2 ml. water, then eluted several times with 5 per cent aq. pyridine. The eluates were dried in the cold and subjected to paper electrophoresis. With regard to the quantitative aspect of the analysis of the hydrolysate, it was found that when glucose, glucose monosulfate and cellobiose determinations were performed on the eluates from the charcoal, respective 76 per cent, 72 per cent and 94 per cent recoveries of the initial sugars were obtained.

Determination of the Position of the Sulfate on Glucose—Glucose monosulfate was oxidized by periodic acid and formaldehyde produced was measured colorimetrically by the chromotropic acid-formaldehyde reaction. This method was based on the procedures described by Burton for the determination of glycerol (*6*) and by Suzuki and Strominger for the determination of glucosamine (*7*). Glucose monosulfate fraction separated by paper electrophoresis was eluted with water from the filter paper. To this sample solution which contained about 0.05 μmoles of glucose per 0.01 ml. and 0.05–0.1 μmoles of glucose monosulfate and which was adjusted to 0.03 ml. with water were added, 0.005 ml. of 5 *M* acetate buffer pH 4.5 and 0.025 ml. of 0.05 *N* sodium periodate. The reaction mixture was kept in the dark at room temperature for 24 hours. Then, 0.03 ml. of 0.05 *N* sodium arsenite was added to it and the reaction mixture was kept in the dark for an additional 15 minutes. Then, it was diluted by addition of 0.16 ml. of water and 1.5 ml. of chromotropic reagent, and heated on a water bath for 30 minutes at 100°. It was cooled to room temperature and determined the optical density at 570 mμ. The unknown glucose monosulfate sample always was analyzed simultaneously with blank and standard glucose samples.

EXPERIMENTALS AND RESULTS

Formation of Cellulosesulfate (*S-poor*)—S-poor cellulosesulfate was obtained from the digests of cellulosepolysulfate by cellulosepolysulfatase contained in "preparation B". In the experiment, 15 mg. of cellulosepolysulfate was

TABLE I

Formation of S-poor Cellulosesulfate from S-rich Cellulosepolysulfate with Cellulosepolysulfatase

Reaction mixture contained 5 ml. enzyme solution ("preparation B") which was adjusted to pH 6.0 with 0.1 *M* acetate buffer and 15 mg. cellulosepolysulfate. A few drops of chloroform were added and the reaction mixture was incubated at 37°. After incubation period, the reaction mixture was boiled and centrifuged, and the supernatant was throughly dialyzed. Ester sulfate and total sugar (estimated as glucose) in 0.5 ml. of the non-dialyzable product were determined.

Time (*hours*)	Total sugar *μmoles* (estimated as glucose)	Ester sulfate *μmoles*	$\frac{\text{Ester sulfate}}{\text{glucose}}$
15	1.85	2.97	1.6
15	1.22	1.85	1.5
30	1.25	0.81	0.65
45	1.02	0.56	0.55
45 (a)	1.50	4.15	2.77

a) with boiled enzyme

incubated for 1-2 days with 5 ml. of "preparation B" adjusted to pH 6.0 with 0.1 *M* acetate buffer, in the presence of a few drops of chloroform. No increase of reducing power was detected during the incubation. The reaction mixture was boiled and centrifuged. The supernatant was throughly dialyzed to remove inorganic sulfate. Ester sulfate and total sugar (estimated as glucose) in the non-dialyzable product were determined. By varying the incubation time, several products were obtained with the ratio of ester sulfate to glucose between 1.6 and 0.5. (Table I).

The evidence which suggested that cellulosepolysulfatase action preceded that of polysaccharases was obtained thus:

1. The effect of some common inhibitors on the two enzymes (cellulosepolysulfatase and polysaccharases) in the crude extract was tested. It can be seen from Table II that fluoride, *p*-chloromercuribenzoate and maleic acid were powerful inhibitors for polysaccharases, but were less effective on cellulosepolysulfatase. Indeed, in the presence of 0.02 *M* fluoride, S-poor cellulosesulfate was obtained (*1*). It is clear that cellulosepolysulfatase activity is not dependent on preliminary degradation of polysaccharide, on the other hand, none of the compounds tested selectively inhibited cellulosepolysulfatase.

TABLE II

Effect of Inhibitors on the Cellulosepolysulfatase and Polysaccharases in the Crude Liver Extract

Inhibitors were allowed to act as shown in the Table and the residual enzyme activities were measured against a 0.3 per cent solution of potassium cellulosepolysulfate in the presence of 0.1 *M* acetate buffer, pH 5.2. Incubation was for 3 hours at 37°.

Inhibitor	Concn. (*M*)	Inhibition (%)	
		Cellulosepoly-sulfatase	Polysaccharases
No inhibitor		0	0
KH_2PO_4	10^{-3}	3.7	
NaF	2×10^{-2}	35.6	100
KCN	2×10^{-3} 2×10^{-2}	6 0	
Ferricyanide	2×10^{-3}	0	
Maleic acid	2×10^{-3}	23	47
p-Chloromercuribenzoate	10^{-3}	8.5	62
Thiourea	2×10^{-2}	10	
8-Hydroxyquinoline	5×10^{-4}	0	
EDTA	2×10^{-3} 2×10^{-2}	0 13	
Anilin	2×10^{-2}	2.5	

2. It can be seen from Table III that sulfatase-free polysaccharases were very active towards cellulosesulfate (S content 6.8 per cent, ester sulfate/glucose=0.5), but less active towards cellulosepolysulfate (S content 18.0 per cent, ester sulfate/glucose=2.7). These findings are compatible with the possibility that some preliminary release of inorganic sulfate from the cellulosepolysulfate is necessary prior to the possible enzymatic degradation of the polysaccharide chain.

TABLE III

Polysaccharase Activity towards Cellulosepolysulfate and Cellulosesulfate

Reaction mixture contained 0.5 ml. enzyme solution and substrate (1 mg. as glucose), and adjusted to 1 ml. in the presence of 0.1 *M* acetate buffer pH 6.0. A few drops of chloroform were added and the reaction mixture was incubated at 37°. After incubation period, the reducing power was determined after deproteinization with zinc hydroxide gel.

	Liberated reducing power (%) estimated as glucose for the complete hydrolysis	
Substrates / Time (*hours*)	Cellulosepolysulfate S content 18.0%	Cellulosesulfate S content 6.8%
14	0	19.2
39	0.8	44.0
72	1.0	48.0

Formation of Glucosesulfates; (*A*) *By the Crude Liver Extract*—In the digestion of cellulosepolysulfate by the crude liver extract, intermediates of the reaction were obtained as follows: Final volume of reaction mixture was 50 ml. containing 25 ml. of crude liver extract and 150 mg. of cellulosepolysulfate in the presence of 0.01 *M* acetate buffer pH 5.2, and the reaction was carried out in the cellophane bag dialyzing against 300 ml. 0.01*M* acetate buffer in the presence of a few drops of chloroform with stirring for 2 days at room temperature. Dialyzable substances were purified by the charcoal adsorption. The three distinct components were then detected by means of paper electrophoresis of the eluate (citrate buffer pH 3.5, 0.02 *M* 300 volt./40 cm., 2 hours).

The principal component (about 400 μg. estimated as glucose) was identified with glucose.

The second component (about 160 μg. estimated as glucose by the anthrone method), bearing one mole of sulfate per glucose unit, had the same R_f as glucose monosulfate on paper chromatogram (solvent: glacial acetic acid, *n*-butanol, water, 15 : 50 : 35) and in addition, gave a reducing power close to glucose monosulfate. These facts indicated that the second component was glucose monosulfate. In order to determine the position of the sulfate on glucose, the oxidation of the glucose monosulfate with periodate was carried out. One mole of formaldehyde was formed from one mole of glucose monosulfate. The result indicates that the sulfate is not attached to the 6-position of glucose.

The third component (about 25 μg. estimated as glucose) giving three moles of sulfate per glucose unit, might be regarded as a trisulfated glucose.

Charoninsulfate, prepared from the mucous gland of *Charonia lampas* and consisted mainly cellulosepolysulfate (*8*), was also digested with crude liver extract by the same method to yield glucose monosulfate as one of the intermediates.

(*B*) *By the "preparation B"*—In other hand, cellulosesulfate (S-poor), obtained from cellulosepolysulfate (S-rich) by the above method with "preparation B", was freeze-dried and further digested with purified polysaccharase preparation. For the identification of the reaction products, the charcoal adsorption was used. On the paper electrogram, glucose and glucose monosulfate were identified. In order to determine the position of the sulfate on glucose, the oxidation of the glucose monosulfate with periodate was carried out. No formaldehyde was formed from glucose monosulfate. The result indicates that the sulfate is attached to the 6-position of glucose.

DISCUSSION

The occurrence of sulfatases hydrolyzing sulfate ester bonds of polysaccharide sulfates without preliminary degradation of polysaccharide moiety has never been confirmed (*9*). For example, chondrosulfatase of bacteria catalyzes the hydrolysis of chondroitinsulfate only after appropriate degradation of the polysaccharide chain (*10*). The cellulosepolysulfatase is the first example of polysaccharide sulfatase. It is certain that the polysaccharase fraction includes cellulase, since it hydrolyzes CM-cellulose and cellulosesulfate (S-poor) to the same degree. These results led us to consider that the digestion of cellulosepolysulfate by the enzyme preparation proceeds as shown in the scheme mentioned above.

By the stepwise digestion of cellulosepolysulfate with cellulosepolysulfatase and cellulase, as shown in the experimental, glucose 6-sulfate was obtained as the final product. It may be explained by the assumption that cellulosepolysulfatase splits preferentially 2- and 3-sulfate ester bonds. On the contrary, by the simultaneous attack with cellulase and sulfatases in the crude enzyme preparation, glucose 2- and 3-sulfates were identified as the intermediates. It may be explained by the predominant action of cellulase and glucosulfatase which splits preferentially 6-sulfate ester bond.

According to Soda and Egami (*11*), the liver extract of *Charonia lampas* contains also a highly active chondrosulfatase. However it could not be confirmed. It remains to be elucidated, whether it is due to some seasonal variations, ecological conditions or any other reasons.

Charoninsulfate was also digested with crude liver extract to yield glucose monosulfate as one of the products. It should be emphasized that both charoninsulfate and the enzymes were isolated from the same animal.

Therefore, charoninsulfate may be regarded as at least one of the natural substrates of these enzymes in *Charonia lampas*.

SUMMARY

1. Cellulosepolysulfate was digested with cellulosepolysulfatase of the liver of *Charonia lampas* free from polysaccharases. Thus S-poor cellulosesulfate was obtained.

2. After further digestion of the S-poor cellulosesulfate with polysaccharases, glucose 6-monosulfate and glucose were obtained.

3. In the digestion of cellulosepolysulfate by the crude liver extract, when the reaction was carried out in the cellophane bag dialyzing against acetate buffer, glucose 2- and/or 3-monosulfate and a small amount of glucose trisulfate were obtained as the intermediates.

The author thanks Prof. F. Egami for his interest and encouragement. A part of the expense of this study was defrayed by a grant from the Ministry of Eduction and from Seikagaku-Kenkyusho Ltd. to Prof. Egami, to which her thanks are due. Some of the experiments were carried out in the Marine Biological Institute of Nagoya University.

REFERENCES

(*1*) Takahashi, N., and Egami, F., *Biochim. et Biophys. Acta*, **38**, 375 (1960)
(*2*) Takahashi, N., *J. Biochem.*, **48**, 508 (1960)
(*3*) Scott, T. A., and Jr., Melvin, E. H., *Anal. Chem.*, **25**, 1656 (1953)
(*4*) Somogyi, M., *J. Biol. Chem.*, **160**, 61 (1945)
(*5*) Rapport, M., Meyer, K., and Linker, A., *J. Am. Chem. Soc.*, **73**, 2416 (1951)
(*6*) Burton, R. M., "Methods in Enzymology" vol. III. ed. by Colowick, S. P. and Kaplan, N. O., Baltimore, p. 246 (1957)
(*7*) Suzuki, S., and Strominger, J. L., *J. Biol. Chem.*, in press
(*8*) Egami, F., Asahi, T., Takahashi, N., Suzuki, S., Shikata, S., and Nisizawa, K., *Bull. Chem. Soc. Japan*, **28**, 685 (1955)
(*9*) Beuhler, H. J., Katzman, P. A., and Doisy, E. A., *Proc. Soc. Exptl. Biol. N. Y.*, **78**, 3 (1951), Reggianini, O., *Boll. Soc. Ital. Biol. Sper.*, **26**, 1117 (1950), Korn, E. D., *J. Biol. Chem.*, **226**, 841 (1957)
(*10*) Dodgson, K. S., and Lloyd, A. G. *Biochem. J.*, **66**, 532 (1957), Konetza, W. A., Pelczar, M. J., and Burnett, G. W., *Bact. Proc.*, 37 (1954)
(*11*) Soda, T., and Egami, F., *J. Chem. Soc. Japan*, **59**, 1202 (1938)

The Journal of Biochemistry, Vol. 48, No. 5, 1960

CHEMICAL MODIFICATION OF TUBERCULIN PROTEIN

II. DIAZOTIZATION

By YOSHIMI OKADA

(*From the Department of Biochemistry, Faculty of Science,*
*Osaka University, Osaka**)

(Received for publication, May 12, 1960)

In the previous paper (*1*) the author reported the chemical modifications of tuberculin-active protein, named hC, with 2, 4-dinitrofluorobenzene (DN-FB) and *p*-phenylazobenzoylchloride (PABC). It was suggested that at least tyrosine residues and half of the lysine residues are not directly concerned in the tuberculin activity.

In the present work, aromatic diazonium compounds were chosen as the modifying reagents. The coupling reaction of aromatic diazonium compounds of proteins has been used to some extent in enzymic studies (*2*) to determine requirements for histidyl and tyrosyl residues for activity. Recently Okamoto and coworkers (*3*) applied this coupling reaction to culture media of tubercle bacilli and prepared *o*-aminophenol azo tuberculin (OA-Azo-T), which was as potent as Purified Protein Derivative (*4*). It was suggested from this result that tyrosyl and/or histidyl groups may not be essential for tuberculin activity. However, it has not been demonstrated directly that tyrosine and/or histidine groups in OA-Azo-T were diazotized completely. On the other hand, other groups of the protein may also react with aromatic diazonium compounds, such as the ε-amino group of lysine (*5, 6*). In order to correlate the chemical structure of tuberculin active protein with the biological activity, it was of interest to investigate the coupling of diazonium compounds with tuberculin active protein quantitatively and then to obtain a more precise description of the groups derived. In the present investigation, *o*-aminophenol, *p*-aminophenol, sulfanilic acid and *p*-aminoacetophenone were chosen as the aromatic amine. The result suggested that the coupling reaction of aromatic diazonium compounds with the protein was a complex one, and it has not been established by this method wheather tyrosyl and/or histidyl groups may be responsible for biological activity or not. Both fully and less active diazotized tuberculin proteins were obtained.

---

* Present Address, Department of Biochemistry, Faculty of Medicine, Kyushu University, Fukuoka.

EXPERIMENTAL

MATERIALS

Tuberculin Protein—The tuberculin protein, named hC, was prepared from the culture filtrate of human strain Aoyama B according to the method described by Seibert (*7*) with slight modifications (*1*).

Five hundredth micrograms of hC is potent as 0.1 ml. of Old Tuberculin (×2000 dilution) as reported in the previous paper (*1*).

Chemical Reagents—Mono-(*p*-azoacetophenone)-carbobenzoxytyrosine (AAP-Cbz-Tyr) and mono-(*p*-azoacetophenone)-carbobenzoxyhistidine (AAP-Cbz-His) were kindly supplied by Dr. K. Sugae, Osaka University. All other Chemicals were purchased commercially.

METHODS AND RESULTS

Biological Test—Each 0.1 ml. of the solution to be tested and standard tuberculin (0.5 μg. hC per ml. of physiological saline solution) were injected intracutaneously into the same person simultaneously. Skin reaction was measured at 24 and 48 hours after the injection. In this report are recorded only the 48 hours measurings.

Preparation of Diazo-hC—(a) *o*-Aminophenol azo hC (*o*-APA-hC)—Solution A; Ten ml. of 0.2 M $NaHCO_3$ was added with stirring to 200 mg. of hC dissolved in 170 ml. of 0.05 M Na_2HPO_4 and the mixture was allowed to stand at 5°. Solution B; One hundred and nine mg. of *o*-aminophenol suspended in 10 ml. of 0.2 N HCl was added with stirring to 70 mg. of $NaNO_2$ dissolved in 10 ml. of water. Solution B was added over a period of 10 minutes with continuous stirring to solution A at 5°. After 20 hours at 5° the reaction mixture was acidified slowly with 0.5 N HCl to pH 3.0 and the resulting precipitate was collected and washed with diluted acid. The precipitate was redissolved and reprecipitated twice from water by bringing the pH up to 8.5 and then acidifying slowly. The precipitate finally formed was washed with alcohol until the supernatant solution became colourless and then dried with ether. The product thus obtained was orange coloured powder and designated as *o*-APA-hC. The yield was about 170 mg.

o-APA-hC is soluble in neutral and alkaline solution but insoluble in acid solution as well as hC. The absorption spectrum of *o*-APA-hC in 0.1 N NaOH is shown in Fig. 1. No specific and definite absorption maximum was observed.

(b) *p*-Aminophenol azo hC (*p*-APA-hC)—The procedure was exactly as the described for the preparation of *o*-APA-hC, except that 109 mg. of *p*-aminophenol dissolved in 10 ml. of 0.2 N HCl was added to 70 mg. of $NaNO_2$ dissolved in 10 ml. of water. The yield was about 180 mg.

The product thus obtained and designated as *p*-APA-hC is soluble in neutral and alkaline solution but insoluble in acid solution. The absorption spectrum of *p*-APA-hC in 0.1 N NaOH is shown in Fig. 1. Absorption spectrum of *p*-APA-hC is very similar to that of *o*-APA-hC.

(c) *p*-Aminoacetophenone azo hC (AAA-hC)—Solution A was the same as that described for the preparation of *o*-APA-hC. Solution C; One hundred and thirtyfive mg. of *p*-aminoacetophenone dissolved in 10 ml. of 0.2 *N* HCl was added with stirring to 70 mg. of $NaNO_2$ dissolved in 10 ml. of water. Solution C was added over a period of 10 minutes with continuous stirring to solution A at 5°. After 20 hours at 5°, the reaction mixture was acidified slowly with 0.5 *N* HCl to pH 3.0 and the resulting precipitate was collected and washed with dilute acid until the washings became colourless. The precipitate was washed with alcohol and acetone until the supernatant solution became colourless and then dried with ether. The product thus obtained was red-orange powder and designated as AAA-hC. The yield was about 150 mg.

AAA-hC insoluble in acid and neutral solution, partially soluble in weak alkaline solution (pH 8–10) and soluble in 0.1 *N* NaOH. The absorption spectrum of AAA-hC in 0.1 *N* NaOH is shown in Fig. 1. The absorption maximum was observed at 330 mμ.

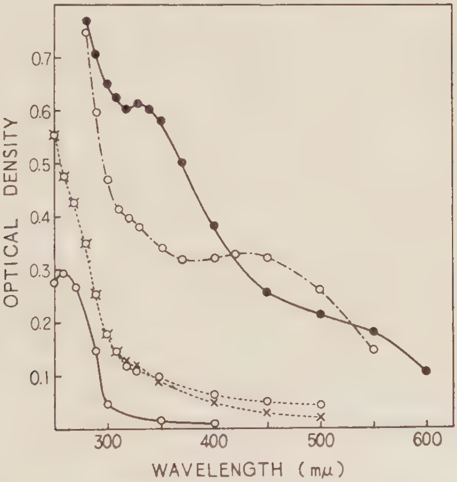


Fig. 1. Absorption spectra of hC, and Diazo-hC's in 0.1 *N* NaOH.

—○— hC, --○-- *o*-APA-hC, --×-- *p*-APA-hC, —●— AAA-hC, -—○—- SAA-hC, each 0.1 mg. per ml.

(d) Sulfanilic acid azo hC (SAA-hC)—Solution A was the same as that described for the preparation of *o*-APA-hC. Solution D; Two hundred and nine mg. of sulfanilic acid was dissolved in 1.0 ml. of *N* NaOH and diluted to 8.0 ml. with water. Seventy milligrams of $NaNO_2$ dissolved in 10 ml. of water was added and then 2 ml. of *N* HCl was added with stirring. Solution D was added over a period of 10 minutes with stirring to Solution A at 5°. After 20 hours at 5°, 50 ml. of 20 per cent trichloroacetic acid solution was added with continuous stirring and the resulting precipitate was collected, washed with 4 per cent trichloroacetic acid solution and dissolved in 50 ml. of 0.05 M Na_2HPO_4. The solution was treated again with trichloroacetic acid solution. This procedure was repeated two times and the precipitate finally formed was washed with alcohol and ether. The product thus obtained and designated as SAA-hC was yellow-orange powder. The yield was about 185 mg.

SAA-hC is soluble in neutral, alkaline and also dilute acid solution. The absorption spectrun of SAA-hC in 0.1 N NaOH is shown in Fig. 1. The absorption maximum and plateau were observed at 420 mμ and 330 mμ, respectively.

Tuberculin Activity of Diazo-hC's—Tuberculin activity of diazotized-hC's are shown in Table I. *o*-APA-hC and *p*-APA-hC were as potent as unmodified hC corresponding to that reported by Okamoto *et al.* (*3*), but APA-hC and SAA-hC were partially inactivated.

The variation of the rate of inactivation could be readily explained if different kinds and/or different extent of amino acid residues in hC molecule were reacting with different diazonium compounds. In an attempt to shed some light on the variation of the rate of inactivation, amino acid analyses were performed upon diazo-hC's.

TABLE I

Tuberculin Activity of Diazo-hC's

Diazo-hC	Dose	Number of person tested	Averag dimension of reaction (mm.)	
			Diazotized-hC	hC
o-APA-hC	0.05	18	22×26	23×28
p-APA-hC	0.05	15	21×25	21×27
AAA-hC	0.05	21	12×15	21×24
SAA-hC	0.05	18	13×15	23×28

Amino Acid Analyses—Particular attention was paid to aromatic and basic amino acids which were known to react with aromatic diazonium compounds. All analyses were performed with 15 cm. column of Dowex-50 according to the method described by Moore and Stein (*7*). This procedure gave us the value for the sum of tyrosine and phenylalanine. The tyrosine concentration was determined colorimetrically with Folin reagent (*8*) and the phenylalanine concentration was calculated by substracting the tyrosine value from the sum of tyrosine and phenylalanine obtained chromatographically. Hydrolysis was conducted by heating about 50 mg. of protein in 10 ml. of 6 N HCl at 110° for 24 hours in sealed tubes.

(a) hC—The aromatic and basic amino acid contents were given in Table II and a typical elution curve is presented in Fig. 2a.

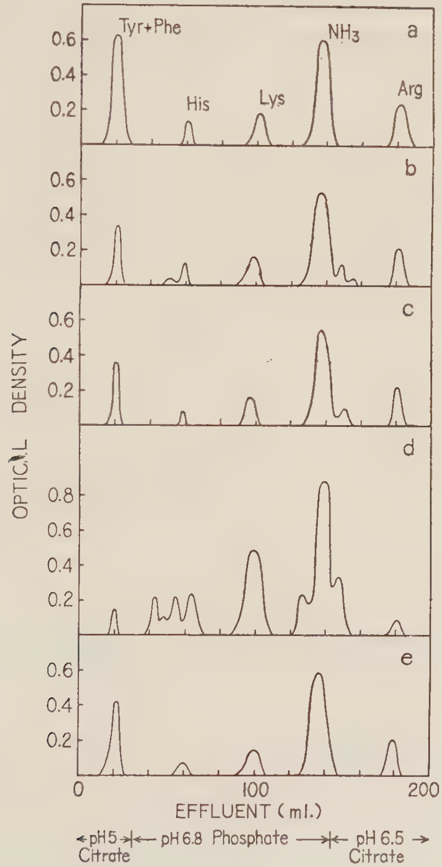


FIG. 2. Aromatic and basic amino acid analyses of hC and diazo-hC's.

The acid hydrolysates were analyzed using columns of Dowex 50 (*7*). (a) hC, (b) *o*-APA-hC, (c) *p*-APA-hC, (d) AAA-hC, (e) SAA-hC.

(b) *o*-APA-hC—As can be seen from Table II, phenylalanine, arginine and lysine contents of *o*-APA-hC were recovered quantitatively, whereas recovery of histidine and tyrosine contents was about two third of the content of original protein. It was demonstrated that only one third of tyrosine and histidine residues in hC was modified with *o*-aminophenol azo compound under the condition employed.

In each of the chromatograms obtained from hydrolysates of diazo-hC's, peaks appear that are not normally seen with hydrolysate of unmodified hC. In the hydrolysate of *o*-APA-hC (Fig. 2b), new peaks appeared, one at 145 ml., the position after ammonia and another at 55 ml., ahead of histidine. The former peak was found also with the hydrolysate of *p*-APA-hC and AAA-hC. It seems probable that this new peak was attributable to a product formed by the acid decomposition of diazoderivatives of tyrosine mainly (see AAA-hC).

TABLE II

Contents of Aromatic and Basic Amino Acids in hC and Diazo-hC

	Residues of amino acid per 10^5 g. protein				
	hC	*o*-APA-hC	*p*-APA-hC	AAA-hC	SAA-hC
Tyr	14.0	10.5	11.0	4.8	8.0
Phe	13.4	12.8	14.0	4.5	12.9
Arg	30.8	31.0	30.2	7.8	30.5
His	12.4	9.5	8.9	—2)	6.0
Lys	24.8	25.8	25.2	—2)	18.2

1) The amount of original hC in the Diazo-hC's was determined by estimation of the amide content of the both proteins.

2) As can be seen from Fig. 2, new components were overlapping at these amino acids.

(c) *p*-APA-hC—As can be seen from Table II, the analytical data for the hydrolysate of *p*-APA-hC are almost the same as those obtained from *o*-APA-hC.

(d) AAA-hC—As can be seen from the results of the analysis tabulated in Table II, the contents of tyrosine, phenylalanine, and arginine in the AAA-hC are only about 4.8, 4.5 and 7.8 residues, whereas unmodified hC yields 14.0, 13.4 and 30.8 residues, respectively, per 10^5 g. of protein. As can be seen from Fig. 2d, several new components appeared, some of which are indistinguishable from histidine and lysine, one at the 145 ml. is at the same position observed in the hydrolysate of the *o*-APA-hC and *p*-APA-hC.

In an attempt to shed some light on the complex effluent curve, paper chromatographic studies for the acid hydrolysate of AAP-Cbz-Tyr and AAP-Cbz-His were performed as model experiments by the usual method using Toyo No. 50 filter paper and *n*-butanol-acetic acid -water (4 : 1 : 5) as mobile phase. The compound was detected with a ninhydrin spray reagent. The result obtained is shown in Fig. 3. It is evident that AAP-Cbz-Tyr and AAP-Cbz-His were completely decomposed by acid hydrolysis without regeneration of tyrosine and histidine. Three major spots are found as artifact in the hydrolysate of AAP-Cbz-Tyr and no definite spots are observed in the hydrolysate of AAP-Cbz-His. No attempt was made to separate the components of the decomposition products in quantities suitable for further characterization.

The recovery of arginine and phenylalanine is very low. The coupling reaction of diazonium compounds with arginine and phenylalanine residues might not be expected on the basis of experience. For example, the recovery of arginine and phenylalanine is quantitative in hydrolysates of other three diazotized hC as can be seen in Table II. It is probable that secondary changes taking place during the hydrolysis make the decomposition of arginine and phenylalanine residues. For example, if AAA-hC (30 mg.) was added to arginine (3 mg.) immediately before hydrolysis, analysis of the hydrolysate by the Sakaguchi reaction showed that about one-third of arginine had been lost. It is likely that some of the new peaks were also attributable to the decomposition products of arginine and other amino acids. Detailed sttdy of amino acid analysis of AAA-hC is complicated by the multiplicity of reaction process and products. It is impossible, therefore, to compare the results obtained from the hydrolysate of AAA-hC with that of other diazo-hC.

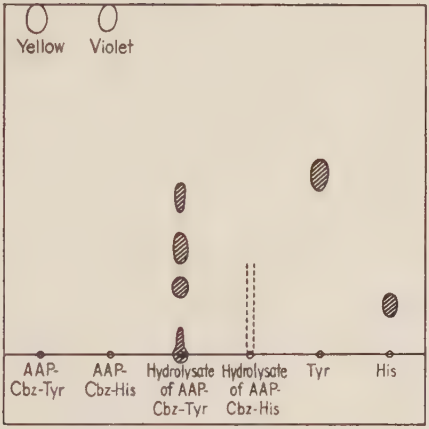


FIG. 3. Paper chromatogram of AAP-Cbz-Tyr, AAP-Cbz-His and their acid hydrolysate.

(e) SAA-hC—As can be seen from Table II, the recovery of tyrosine, histidine and lysine was low. It was reported that ε-amino group of lysine may also react with aromatic diazonium compounds although this has not been demonstrated directly (*5*, *6*). Many new peaks as can be seen in the hydrolysate of AAA-hC were not found in the hydrolysate of SAA-hC. It is probable, therefore, that the low recovery of lysine is attributable to diazotization of ε-amino group of lysine.

DISCUSSION

From the data reported in the present communication, it is clear that the extent of the coupling reaction of aromatic diazonium compound with hC is a function of aromatic amine and it is complicated by various factors. Apparently tyrosine and histidine residues in hC were diazotized by four diazonium compounds in different extents, but no evidence on the complete diazotization of these residues was obtained on any the diazotized hC's. Therefore, it is impossible to conclude that tyrosine and histidine residues in tuberculin protein are not essential for the activity as mentioned by Okamoto *et al.* (*3*). As can be seen from Table II hC was diazotized by *o*- and *p*-aminophenol diazonium compounds, to almost the same extents, only the third of tyrosine residues and one fourth of histidine, and it was also demonstrated that the tuberculin activity of the modified protein remained unaffected as indicated in Table I. From the data, therefore, it is only possible to say that one third of tyrosine and one fourth of histidine are not directly concerned in the activity. Tuberculin activity of SAA-hC, in which about half of tyrosine and histidine residues and one fourth of lysine were diazotized, was partially inactivated. In the previous paper (*1*), it was reported that tuberculin activity was not affected even after half of lysine residues and all of the tyrosine residues were blocked by 2, 4-dinitrophenyl group. Thus, as a possible hypothesis, it is probable that histidine residues, which are diazotized by sulfanilic acid diazonium compound, are partially concerned in the activity. At the same time, it seems likely that solubility of tuberculin protein is in some way concerned in biological activity. DNP-hC-S, PAB-hC (*1*), *o*-APA-hC, *p*-APA-hC, and hC, all have the same properties in solubility against pH reaction, *i.e.*, these were soluble in neutral and alkaline solution, but insoluble in acid solution, and all have almost the same tuberculin potency. SAA-hC is soluble in acid solution, while AAA-hC is insoluble in neutral and dilute alkaline solution. Both these latters have different properties in solubility from the other hC derivatives and have decreased activities.

Pauly isolated the reaction product of diazotized arsanilic acid with chloroacetyltyrosine and concluded that the bisazo derivative has been formed based on the elementary analysis (*9*). Recently, Tabachnick and Sobotka (*10*) isolated the product that is characteristic for monoazo-derivative of chloroacetyltyrosine from the reaction mixture according to Pauly's procedure. This product was separated to four bands by chromatography on DEAE-cellulose. It is indicated that the coupling of diazonium compounds with protein is complicated by the multiplicity of reaction.

The different types of amino acid residues in hC react with different aromatic amine diazodium compounds. *o*-Aminophenol and *p*-aminophenol diazonium compounds react with histidine and tyrosine residues in hC partially, but do not react with lysine residues under condition employed. Sulfanilic acid diazonium compound reacts with histidine, tyrosine and also lysine residues in hC under the same condition. Recently, for similar example, it was reported that different types of amino acid residues in the ribonuclease molecule react with iodoacetate at different pH values (*11*). Amino acid analysis showed that at pH 5.6 to 6 histidine residuce is involved, at pH 8.5 to pH 10 the reaction occurs with lysine residue, whereas at pH 2.8 methionine residue is alkylated. On the study of chemical modification of protein it is necessary to give a certain direct evidence of the reaction of reagent with amino acid residues in protein.

It is interesting that mole ratios of aromatic and basic amino acids contents of hC are partially similar to those of crystalline tuberculin active peptide (TAP) prepared by Yamamura *et al.* (*12*). Namely, molar ratios of Phe : Tyr : His : Lys : Arg are about 1 : 1 : 1 : 2 : 2.5 with hC and 1 : 1 : 1 : 2 : 10 with TAP. The structural responsibility for the tuberculin activity might be partially attributed to some particular peptide part having specific amino acid composition.

SUMMARY

1. Tuberculin active protein, hC, was diazotized with several aromatic diazonium compounds. *p*-APA-hC, which was prepared with *p*-aminophenol, and *o*-APA-hC, prepared with *o*-aminophenol, were as potent as unmodified hC. SAA-hC, prepared with sulfanilic acid, and AAA-hC, prepared with *p*-aminoacetophenone, were less potent than hC.

2. Amino acid analysis, particularly about aromatic and basic amino acids, was performed upon hC and diazo-hC's.

3. Mole ratios of several amino acids contents of hC are similar to those of crystalline tuberculin active peptide.

The author wishes to thank Prof. S. Akabori, Osaka University, for his continuous interest and advice during the course of this investigation, and to thank Prof. Y. Yamamura, Kyushu University, for his encouragement and guidance. The author also wishes to thank Dr. K. Ogura, Toneyama Hospital, for his helpful advice, especially on the skin test technique.

REFERENCES

(*1*) Okada, Y., *J. Biochem.*, **48**, 63 (1960)

(*2*) Fraenkel-Conrat, H., Bean, R. S., and Lineweaver, H., *J. Biol. Chem.*, **177**, 385 (1949)

(*3*) Okamoto, H., Kakishita, M., Ito, R., Saito, T., and Koshimura, S., *Japan. J. Tuberc.*, **2**, 228 (1954)

(*4*) Seibert, F. B., and Glenn, J. T., *Am. Rev. Tuberc.*, **44**, 9 (1941)

(*5*) Howard, A. N., and Wild, F., *Biochem. J.*, **65**, 651 (1957)

(*6*) Gelewitz, E. W., Riedman, W. L., and Klotz, I. M., *Arch. Biochem. Biophys.*, **53**, 411 (1954)

(*7*) Moore, S., and Stein, W. H., *J. Biol. Chem.*, **192**, 663 (1951)

(*8*) Folin, O., and Ciocalteu, V., *J. Biol. Chem.*, **73**, 627 (1927)

(*9*) Pauly, H., *Z. Physiol. Chem.*, **94**, 284 (1951)

(*10*) Tabachnick, M., and Sobotka, H., *J. Biol. Chem.*, **234**, 1726 (1959)

(*11*) Gundlach, G., Stein, W. H., and Moore, S., *J. Biol. Chem.*, **234**, 1754 (1959)

(*12*) Yamamura, Y., Morisawa, S., Tanaka, A., and Shojima, K., *Proc. Japan Acad.*, **35**, 295 (1959)

The Journal of Biochemistry, Vol. 48, No. 5, 1960

BIOCHEMICAL STUDIES ON THE SNAKE VENOMS

VII. ISOLATION OF VENOM CHOLINESTERASE BY ZONE ELECTROPHORESIS*

By CHEN-CHUNG YANG, WEN-CHING CHIU
AND KUANG-CHING KAO

(From the Department of Biochemistry, Kaoshiung Medical College, Kaosiung Taiwan)

(Received for publication, May 24, 1960)

An acetylcholine-inactivating enzyme found in cobra venom by Indian workers (*1*) aroused great interest because of the possible relationship to the neurotoxic principle of the cobra poison. Soon after, however, the neurotoxin and the cholinesterase have been separated into two fractions (*2*, *3*). The former displayed no cholinesterase activity and the latter no curare-like action. On the basis of the cholinesterase content, snake venoms seem to fall into two grous, the venoms of the *Elapidae* being characterized by the presence, and the venoms of the *Viperidae* and *Crotalidae* (*2*, *4*, *5*) by the absence, of cholinesterase.

In the experiments with Formosan snake venoms, recently, Chang and Lee (*6*) confirmed the above findings that the cholinesterase is contained only in the venoms of *Elapidae* but not in those of *Viperidae* or *Crotalidae*, and that it is not associated with the curare-like action of venoms. They also proved that the venom cholinesterase possesses the specific characteristic of acetylcholinesterase.

In this study Formosan cobra and Banded krait venoms (*Elapidae*) were subjected to zone electrophoresis and the cholinesterase activity was separated distinctly from the venom toxicity as an electrophoretically single fraction.

EXPERIMENTAL

Materials and Methods

Venom—The venoms used in this study were freshly collected Formosan cobra (*Naja naja atra*, NNA) and Banded krait (*Bungarus multicinctus*, BM) venoms, which were dried up with sulfuric acid in a vacuum desiccator in cold room. They were dissolved in 0.1 *M* veronal-HCl buffer, pH 8.6 immediately before use.

Substrate—Acetylcholine chloride was used as a substrate and it was dissolved in 0.1 *M* Tris (hydroxymethyl) aminomethane-HCl buffer, pH 8.0.

---

* This paper was presented at the 52nd Annual Meeting of the Formosan Medical Association, Taipei, November 21, 1959.

Measurement of Enzyme Activity—The cholinesterase activity was measured according to the method of Hestrin (*7*) with certain modifications. A half ml. of venom solution and 0.1 ml. of 0.15 M $MgCl_2$ solution (0.04 M as a final concentration) were placed in a small test tube. After 3 minutes preincubation at 37° the reaction was initiated by the addition of 1.0 ml. of 0.0064 M acetylcholine chloride solution (0.0032 M as a final concentration). Exactly after 10 minutes incubation at 37° 4.0 ml. of alkaline hydroxylamine solution which was prepared immediately before use by mixing equal volumes of 1.75 N NaOH and 1 M hydroxylamine-HCl solution was added to terminate the reaction. The reaction mixture was transferred into Evelyn phototube. Then 2.0 ml. of hydrochloric acid (HCl, Sp. Gr. 1.18, diluted to 6 volumes) and 2.0 ml. of 0.185 M ferric chloride solutions were added and the developed purplebrown color was determined immediately by Evelyn photoelectric colorimeter with 540 mμ filter. The quantity in μmole of acetylcholine hydrolyzed in 60 minutes was defined as an unit of enzyme activity.

Zone Electrophoresis—The zone electrophoresis was performed on a starch column with a vertical type apparatus described in a previous paper (*8*) and the experimental condition was inscribed in the figure legend. For the elute of each fraction the amount of protein and the cholinesterase activity were measured.

RESULTS AND DISCUSSION

The cholinesterase activity of the Formosan cobra and Banded krait venoms was determined with the results shown in Table I. The enzyme activity obtained with the colorimetric method of Hestrin (*7*) was 272 and 2367 units per mg. for Formosan cobra and Banded krait venoms respectively and the activity of the latter venom was 8.7 times more potent than that of the former's. The result was in good accordance with that of Chang and Lee's (*6*) which was obtained by an electrometric titration method.

A strict linear relationship between cholinesterase activity and incubation time could be obtained during the first 15 minutes for both venoms (Fig. 1). The enzyme activity increased in proportion to the doses of venoms within the amount of 6 and 60 μg. for Banded krait and Formosan cobra venoms respectively (Fig. 2).

The stablility of cholinesterase activity of both venoms in 0.1 M veronal-HCl buffer, pH 8.6 either in the presence or in the absence of $MgCl_2$ (0.04 M) were studied. As shown in Fig. 3-A and 3-B the cholinesterase activity in Banded krait venom was quite stable even in the absence of $MgCl_2$. On the contrary, the enzyme activity in Formosan cobra venom was very unstable and lost almost all of the activity during the preservation under cold in a few days. However, it was fairly stable under the presence of $MgCl_2$ (0.04 M).

The instability of the cholinesterase of cobra venom in aqueous solution has been pointed out by Chang and Lee (*6*). They have also proved that it was dued to the co-existence of the anticholinesterase in the same venom (*9*).

Separation of the Cholinesterase Activity—Separation of the cholinesterase activity in the venom was carried out by zone electrophoresis. The distribution of the enzyme activity in each fraction was surveyed and the results obtained are presented in Fig. 4-A and 4-B. In Formosan cobra venom the cholinesterase activity was found in the fractions No. 34-50 and revealed as an electrophoretically single fraction on the back leg of the 5th protein fraction. The recovery of the enzyme activity was 72.9 per cent and the specific activity in fraction No. 43 increased from 272 to 2908 units per mg. of protein *i.e.*, a 10.7 fold increase from crude venom (Table II). After zone electrophoresic separation the cholinesterase activity became more stable than that of crude venom during the preservation in a cold

TABLE I

Cholinesterase Activity of Formosan Cobra and Banded Krait Venoms

Venoms	Activity[1)	
	Colorimetric method	Electrometric titration method[2)
Formosan cobra	272	360
Banded krait	2367	3120
BM/NNA ratio	8.70	8.67

1) The quantity of acetylcholine in μM destroyed by 1 mg. of venom in 60 minutes.

2) Data of Chang and Lee (*6*).

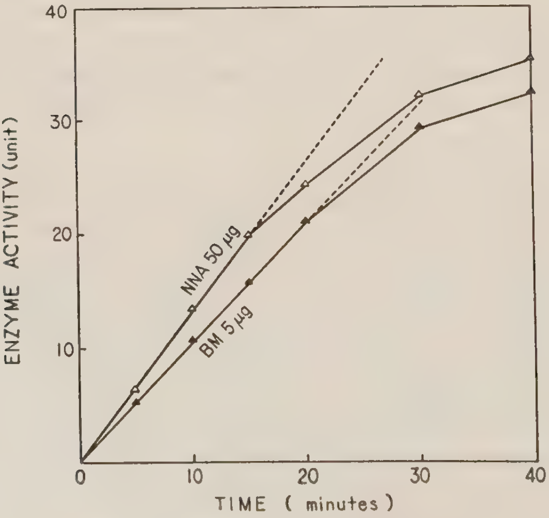


FIG. 1. Time course of the reaction.

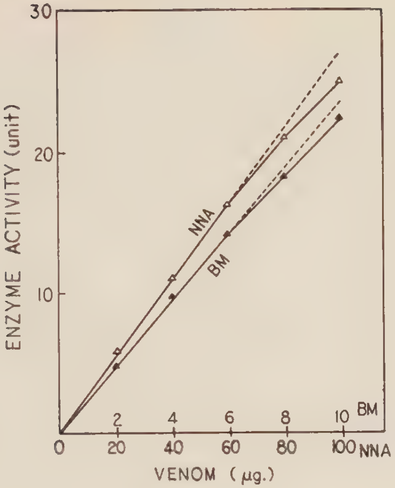


FIG. 2. Enzyme activity plotted as a function of the amount of venoms.

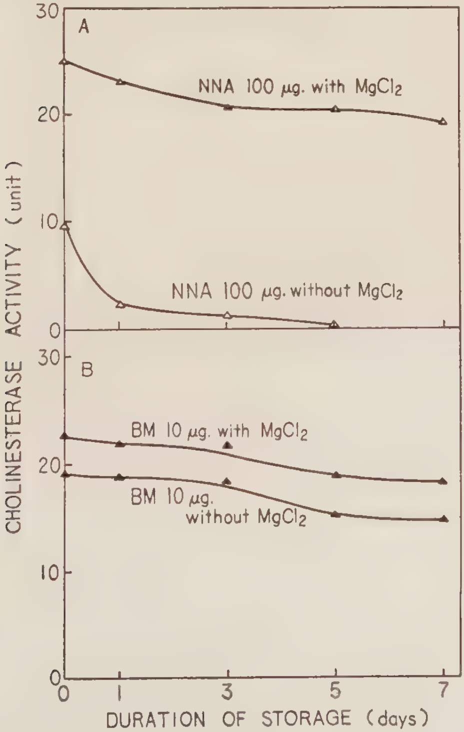
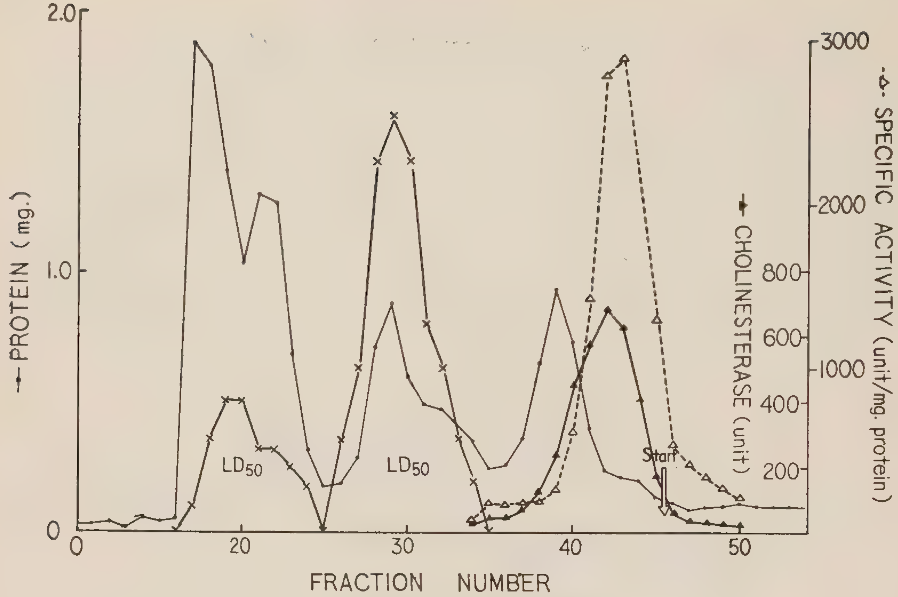
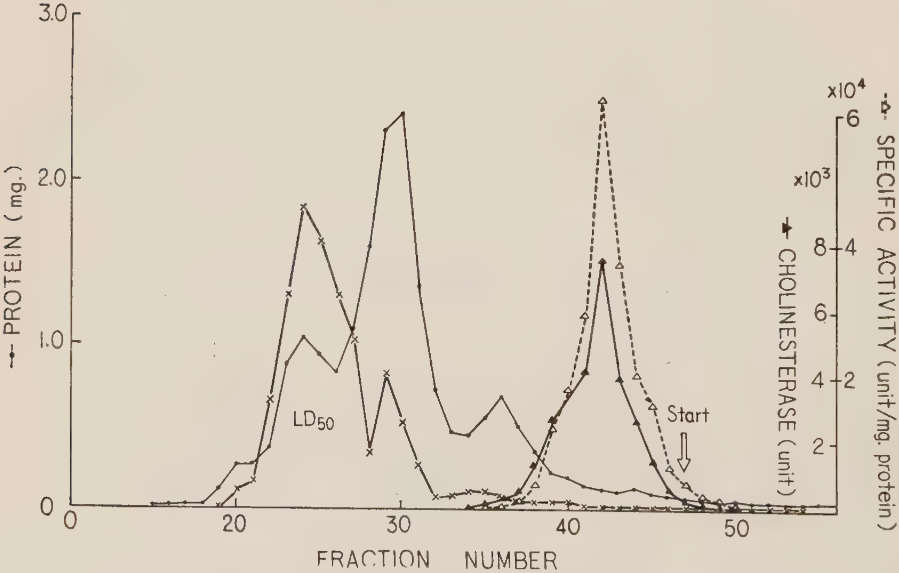


FIG. 3. Stability of the venom cholinesterase activity in 0.1 *M* veronal-HCl buffer, pH 8.6.

A: Formosan cobra venom
B: Banded krait venom



A: Formosan cobra venom



B: Banded krait venom

FIG. 4. Separation of the cholinesterase activity from Formosan *Elapidae* venoms by zone electrophoresis.

20 mg. of dried venom in 1 ml. of veronal-HCl buffer, pH 8.6, ionic strength 0.1, was subjected to electrophoresis in a column 3.5×40 cm. packed with starch. A current of 22 mA (2.3 mA/cm^2., 25.0 volt/cm.) was applied for 24 hours in a cold room (8°). The protein concentration (—·—) of the fraction was determined by Folin's method (*12*) and the protein recovery was 94.9 per cent for Banded krait and 99.1 per cent for Formosan cobra venoms respectively. The cholinesterase activity (—▲—) was measured as described in the text.

TABLE II

Increment of Specific Activity in Each Fraction to Crude (NNA) Venom After Zone Electrophoresis

Fraction Number	Cholinesterase activity (unit)[1]	Protein in mg.	Specific activity	Relative specific activity of each fraction to crude venom
34	25	0.348	72	0.3
35	47	0.244	193	0.7
36	45	0.260	173	0.6
37	67	0.359	187	0.7
38	124	0.649	191	0.7
39	237	0.931	255	0.9
40	450	0.726	620	2.3
41	576	0.400	1440	5.3
42	684	0.244	2803	10.3
43	628	0.216	2908	10.7
44	408	0.196	2082	7.7
45	175	0.134	1306	4.8
46	61	0.112	545	2.0
47	38	0.090	422	1.6
48	35	0.101	347	1.3
49	28	0.101	277	1.0
50	25	0.112	223	0.8
Crude venom	272	1.0	272	1.0

1) The quantity of acetylcholine in μM destroyed in 60 minutes per fraction.

room. It retatined about half of its original activity even after one week storage in the absence of $MgCl_2$. It seems to us that this may be dued to the separation of co-existed anticholinesterase from cholinesterase by zone electrophoresis. In the another trial of zone electrophoresis study we used the buffer solution containing $0.04\,M$ $MgCl_2$ and have increased the recovery of the enzyme activity from 72.9 to 97.2 per cent.

On the attempt to demonstrate the anticholinesterase activity in the fractions obtained by zone electrophoresis the Banded krait venom cholinesterase was incubated with an aliquot of elute in each fraction at 37° for 20 minutes and the cholinesterase activity remaining was measured. No appreciable anti-cholinestrase activity was detected in any fractions obtained after zone electrophoresis. Lee *et al.* (*9*) have suggested that the *Naja* venom anticholinesterase is not a compound of very high molecular weight, such as a protein, since its activity disappears for the most part after dialysis. Therefore it seems to us that the anticholinesterase of *Naja* venom would be some compound of low molecluar weight and has passed through the starch column during the 24 hours electrophoresis.

In Banded krait venom the cholinesterase activity distributed in the fractions No. 35–51 and constituted a high peak between the 3rd protein fraction and the starting point. The recovery of enzyme activity was 62.8 per cent and the specific activity in fraction No. 42 increased from 2367 to 62418 units per mg. of protein, *i. e.*, a 26.4 fold increase from crude venom (Table III) By using the buffer solution containing 0.04 M $MgCl_2$ for the zone electrophoresis separation of the enzyme from Banded krait venom the recovery of the enzyme activity, however, was not improved.

TABLE III

Increment of Specific Activity io Each Fraction to Crude (BM) Venom after Zone Electrophoresis

Fraction Number	Cholinesterase activity (unit)	Protein in mg.	Specific activity	Relative specific activity of each fraction to crude venom
33	18	0.462	39	—
34	46	0.448	103	—
35	152	0.556	273	0.1
36	308	0.688	448	0.2
37	532	0.500	1064	0.4
38	1312	0.344	3814	1.6
39	2736	0.218	12550	5.3
40	3504	0.194	18062	7.6
41	4224	0.142	29747	12.6
42	7615	0.122	62418	26.4
43	3968	0.106	37434	15.8
44	2688	0.132	20363	8.6
45	1456	0.092	15826	6.7
46	576	0.082	6261	2.6
47	268	0.068	3941	1.7
48	128	0.064	2000	0.8
49	75	0.044	1705	0.7
50	33	0.060	550	0.2
51	18	0.044	409	0.2
Crude venom	2367	1.0	2367	1.0

In comparison with another series of our studies (*10*, *11*) it is apparent that the cholinesterase activity of both Formosan cobra and Banded krait venoms separates very distinctly from their toxicity and the result definitely confirmes the conclusion of previous workers that there is no relationship between the cholinesterase activity and the curare-like action of venom.

SUMMARY

Cholinesterase activity in the Formosan cobra and Banded krait venoms has been determined by colorimetric method of Hestrin. The results obtained were in good agreement with that of electrometric titration method. The cholinesterase activity in both Formosan cobra and Banded krait venoms were separated from venom toxicity by zone electrophoresis as an electrophoretically single fraction and the specific activity were increased 10.7 and 26.4 folds respectively. The results proved definitely that there is no relationship between the cholinesterase activity and the venom toxicity.

The authors are gratefully indebted to Prof. R. Hirohata, chief of the Laboratory of Protein Chemistry, Yamaguchi Medical School, Ube, for his continued interest and encouragement during the course of this study.

BIBLIOGRAPHY

(*1*) Iynegar, N. K., Sehra, K. B., Mukerji, B., and Chopra, R. N., *Current Sci.*, India, **7**, 51 (1938)
(*2*) Ghosh, B. N., *Oesterr. Chem.-Ztg.*, **43**, 158 (1940)
(*3*) Sarkar, B. B., Maitra, S. R., and Ghosh, B. N., *Indian J. Med. Research*, **30**, 453 (1942)
(*4*) Ghosh, B. N., Dutt, P. K., and Chowdhury, D. K., *J. Indian Chem. Soc.*, **16**, 75 (1939)
(*5*) Zeller, E. A., *Helv. Chim. Acta.*, **32**, 94 (1949)
(*6*) Chang, C. C., and Lee, C. Y., *J. Formosan Med. Assoc.*, **54**, 103 (1955)
(*7*) Hestrin, S., *J. Biol. Chem.*, **108**, 249 (1949)
(*8*) Yang, C. C., *J. Formosan Med. Assoc.*, **57**, 567 (1958)
(*9*) Lee, C. Y., Chang., C. C., and Kamijo, K., *Biochem. J.*, **62**, 582 (1956)
(*10*) Yang, C. C., Chen, C. J., and Su, C. C., *J. Biochem.*, **46**, 1201 (1059)
(*11*) Yang, C. C., Kao, K. C., and Chiu, W. C., *J. Biochem.*, **48**, 714 (1960)
(*12*) Folin, O., and Ciocalteu, V., *J. Biol. Chem.*, **73**, 627 (1927)

The Journal of Biochemistry, Vol. 48, No. 5, 1960

BIOCHEMICAL STUDIES ON THE SNAKE VENOMS

VIII. ELECTROPHORETIC STUDIES OF BANDED KRAIT (Bungarus Multicinctus) VENOM AND THE RELATION OF TOXICITY WITH ENZYME ACTIVITIES

By CHEN-CHUNG YANG, KUANG-CHING KAO,
AND WEN-CHING CHIU

(*From the Department of Biochemistry, Kaohsiung Medical College,
Kaohsiung, Taiwan*)

(Received for publication, May 24, 1960)

In the series of our work on the snake venoms, Formosan cobra, Hyappoda, Formosan habu, Green habu, and Russell's viper, the venoms were fractionated by zone electrophoresis and the relation between toxicity and enzyme activity were demonstrated (1–4). The only venom remained yet to be studied is the venom of the Banded krait, because of the difficulty of obtaining it.* This venom is the most toxic among the snake venoms in Taiwan, *e.g.*, 16 times more toxic than cobra and more than 100 times than Hyappoda venom.

Therefore, the present study was carried out with extensive effort paid to collecting the appropriate amount of the venom. The venom was subjected to zone electrophoresis in a packed starch column and the distribution of toxicity and of activities of the enzymes, such as phosphomonoesterase, phosphatidase, cholinesterase, L-amino acid oxidase and hyaluronidase was surveyed. The results obtained indicate that the venom toxicity was separated so distinctly from all of the enzyme activities and confirmed definitely that the venom toxicity has nothing to do with any enzyme activities tested.

EXPERIMENTAL

Materials and Methods—Venom of *Bungarus multicinctus* (Banded krait which had been dried over sulfuric acid in a cold room as possible after each collection was pooled and was used in this study. DPN used was a Nutritional Biochemical Corporation product; acetylcholine chloride and L-leucine, a Wakō product. Casein and sodium hyaluronate were isolated from skim milk and an umbilical cord respectively and further purified. The substrates used which are not otherwise specified were the same as described in paper IV of this series (*1*).

Zone electrophoresis, measurement of the enzyme activities and of the toxicity exactly followed the methods used for the study of cobra venom (*1*) and the series of our study on snake venom enzymes (*5–8*). The MLD and LD_{50} of the crude venom used were approximately 1.0 μg. and 0.5 μg. respectively.

---

* As compared with the Formosan cobra of 80 mg. per each extraction, only 1.0–1.5 mg. of venom in dry weight is obtained from each Banded krait.

RESULTS

The elution diagrams of Banded krait venom subjected to zone electrophoresis in a packed starch column are presented in Figs. 1–6. The experimental condition is inscribed in the figure legend and the arrow represents the place where crude venom was applied, *i.e.*, at a height of 24 cm. in the starch stratum. Under this condition, with veronal-HCl buffer, pH 8.6, ionic strength 0.1, the venom migrated toward the cathode and separated into one main protein fraction with two minor protein fractions on its both sides. The main (2nd) protein fraction represents 55.9 per cent of the recovered protein and 23.3 and 17.0 per cent recoveries were observable for both minor protein fractions, the preceding (1st) and the succeeding (3rd) fraction respectively. However, as shown in Fig. 5 the zone electrophoresis pattern of the venom obtained with phosphate buffer, pH 7.6, ionic strength 0.1, was quite different from that obtained with

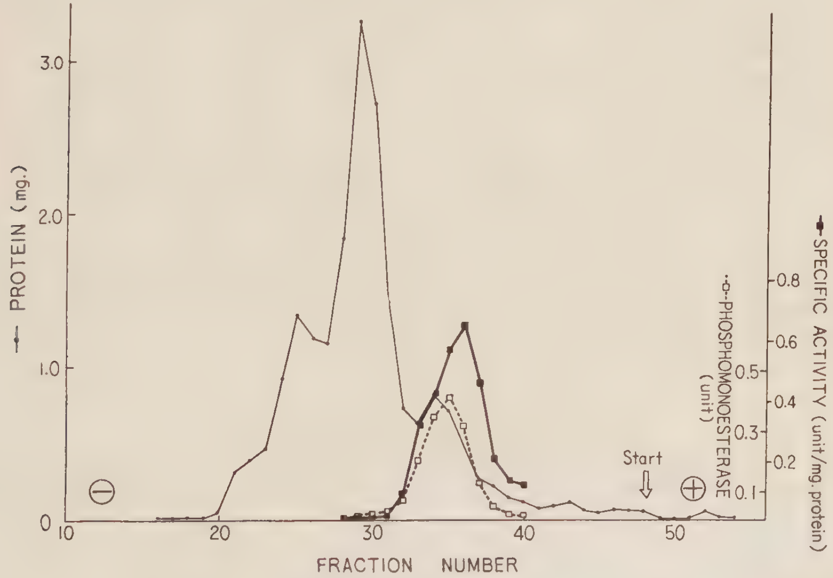


Fig. 1. Zone electrophoretic pattern of Banded krait venom, protein together with phosphomonoesterase activities. 20 mg. of dried venom in Veronal-HCl buffer, pH 8.6, ionic strength 0.1, was subjected to electrophoresis in a column 3.5×40 cm. packed with starch. The experiment was performed in a cold room (7° as measured). A current of 21.2 mA. (2.2 mA/cm^2., 22.9 volt/cm.) was applied for 24 hours. The protein concentration of the fractions was determined by Folin's method (*12*) and protein recovery was 100.2 per cent. Distribution of protein —●—; phosphomonoesterase activities --□--; the specific activities per mg. protein of phosphomonoesterase —□—.

veronal buffer, and revealed only a protein fraction on the cathodic side. The protein recovery was 100.2 per cent in this case.

The distribution of enzyme activities in each fraction was surveyed with the results shown in Figs. 1–5. As Fig. 1 shows, phosphomonoesterase activity was distributed in a rather narrow range and constituted a high peak which coincided well with the 3 rd protein fraction. The recovery of phosphomonoesterase activity was 60.1 per cent and the specific activity in fraction No. 36 increased 4.9 fold from crude venom (Table I). As may be seen from Table II, phosphatase activities other than phosphomonoesterase in Banded krait venom were feebly appreciated, hence their activities in each fraction were unable to be determined.

TABLE I

Specific Activities of Enzymes and of Toxicities per Unit Amount of Protein in Crude Banded Krait Venom and in Eluates after Zone Electrophoresis

Enzymes	Specific activity (unit per mg. protein)		Specific activity increased (fold)
	Crude venom	Eluates	
Phosphomonoesterase	0.132	0.644 (36)[a]	4.9
Phosphatidase A	1060	14302 (35)	13.5
Cholinesterase	2367	62418 (42)	26.4
L-Amino acid oxidase	62.0	933 (36)	15.0
Hyaluronidase	25.6	71.9 (44)	2.8
Toxicity	2000	5602 (23)	2.8

[a] The figures in parentheses denote the fraction number.

TABLE II

Phosphatase Activities of Formosan Snake Venoms

Enzymes / Venoms	Phosphomonoesterase	Phosphodiesterase	5'-Nucleotidase	ATPase	DPN-pyrophosphatase
	unit per mg. of dried venom				
Banded krait	0.132	0.022	6	12	2
Formosan cobra	0.101	1.078	720	580	495
Russell's viper	0.006	3.955	270	227	269
Formosan habu	0.054	3.008	2140	1344	1100
Green habu	0.052	3.116	2320	1620	1190
Hyappoda	0.013	1.382	1211	909	388

The peak of phosphatidase A activity also coincided well with the 3rd protein fraction and revealed that only electrophoretic single fraction is involved in Banded krait venom (Fig. 2). It is the same as in the case of of cobra venom that phosphatidase A activity recovery was more than 100 per cent and amounted to 137.8 per cent.

The data of cholinesterase and L-amino acid oxidase activities in each fraction cited from another series of our works (*5*, *6*) are presented in Figs. 3 and 4. The cholinesterase activity was distributed in the fractions No.

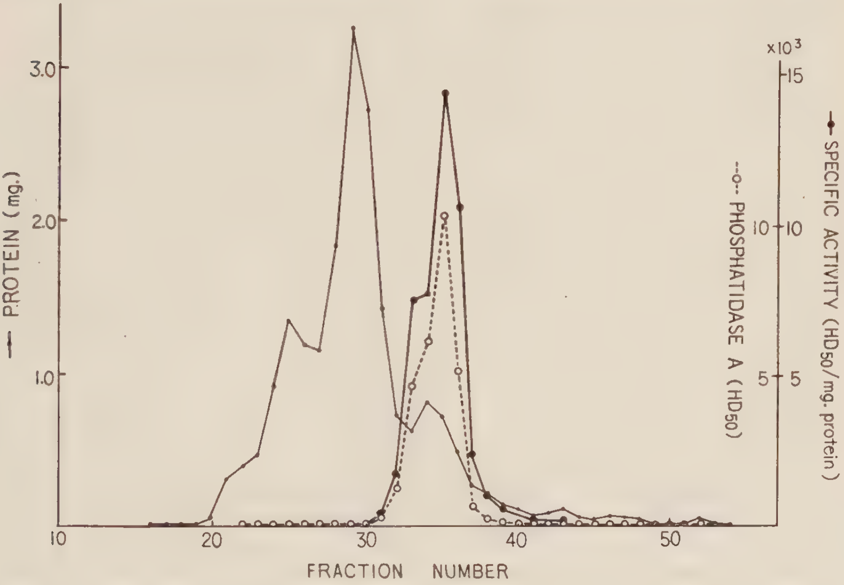


FIG. 2. Distribution of phosphatidase A activities (--○--) and the specific activities (—○—) per mg. of protein in the same electrophoretic run as in Fig. 1.

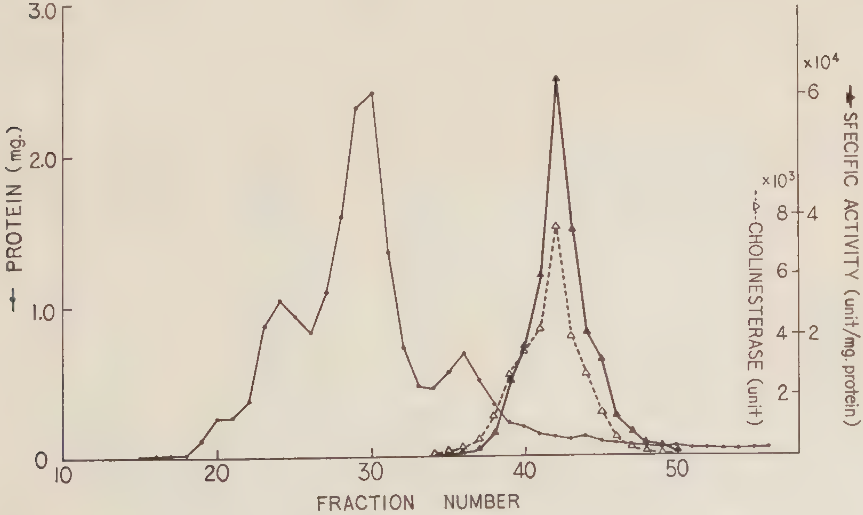


FIG. 3. Distribution of cholinesterase activities (--▲--) and the specific activities (—▲—) per mg. of protein in another electrophoretic run in which the experimental conditions were kept practically the same as in Fig. 1.

33–51 and constituted a high peak between the 3rd protein and the starting point. The recovery of enzyme activity was 62.8 per cent and the specific activity in fraction No. 42 increased from 2367 to 62418 units per mg. of protein, *i.e.*, a 26.4 fold increase from crude venom (Table 1). L-Amino acid oxidase activity was detected only in 7 fractions, *i.e.*, No. 33–39, and separated as an electrophoretically single fraction. The recovery of enzyme activity was 103.2 per cent and the specific activity (Q_{O_2}) increased 15 fold from crude venom.

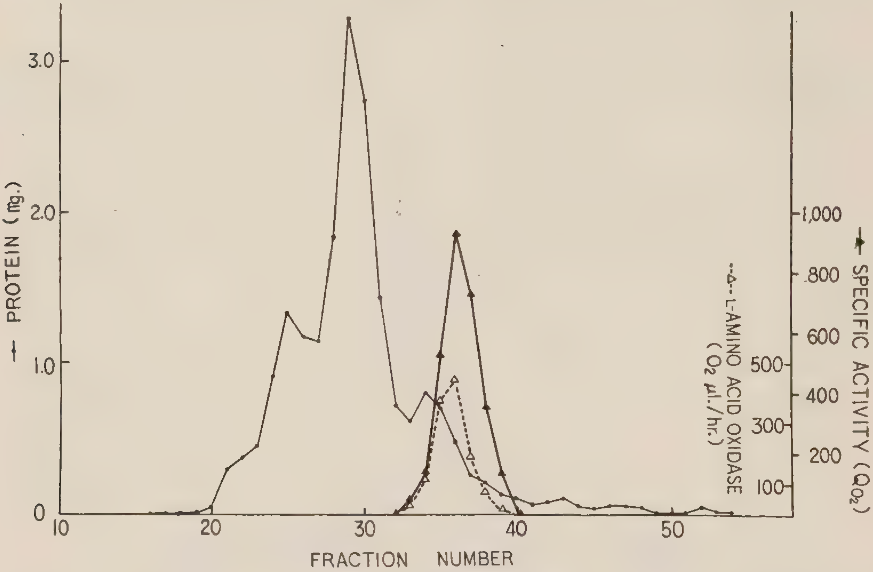


FIG. 4. Distribution of L-amino acid oxidase activities (--△--) and the specific activities (—△—) per mg. of protein in the same electrophoretic run as in Fig. 1.

TABLE III

Enzyme Activities and Toxicities of Formosan Snake Venoms

Enzymes / Venoms	Phosphatidase A (HD_{50})	Cholinesterase	L-Amino acid oxidase (Q_{O_2})	Proteinase $\left([PU]\frac{\text{Cas. FR. A.}}{\mu\text{g. tyr.}}\right)$	Hyaluronidase (T.R.U.)	Toxicity (LD_{50})
	unit per mg. of dried venom					
Banded krait	2193	2367	64.2	0.64	138.9	2000
Formosan cobra	39100	272	40.6	0.68	11.0	125
Russell's viper	245	0	1.8	0.97	31.3	200
Formosan habu	1801	0	385.0	16.2	3.5	36
Green hubu	2473	0	395.0	11.5	29.4	79
Hyappoda	549	0	0	20.0	23.5	18

Since proteinase activity in Banded krait venom is so feeble, 0.64 $〔\mathrm{PU}〕^{\mathrm{Cas.FR.A.}}_{\mu\mathrm{g.tyr.}}$ per mg. of dried venom (Table III), it is impossible to deter-

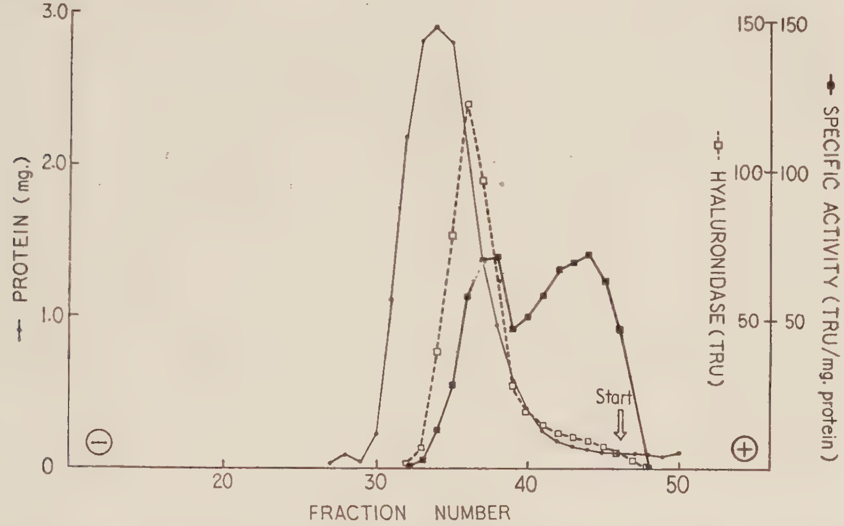


FIG. 5. Distribution of hyaluronidase activities (--■--) and the specific activities (—■—) per mg. of protein in electrophoretic run in which the experimental conditions were kept practically the same as in Fig. 1 and phosphate buffer, pH 7.6. ionic strength 0.1 was used instead of veronal-HCl buffer.

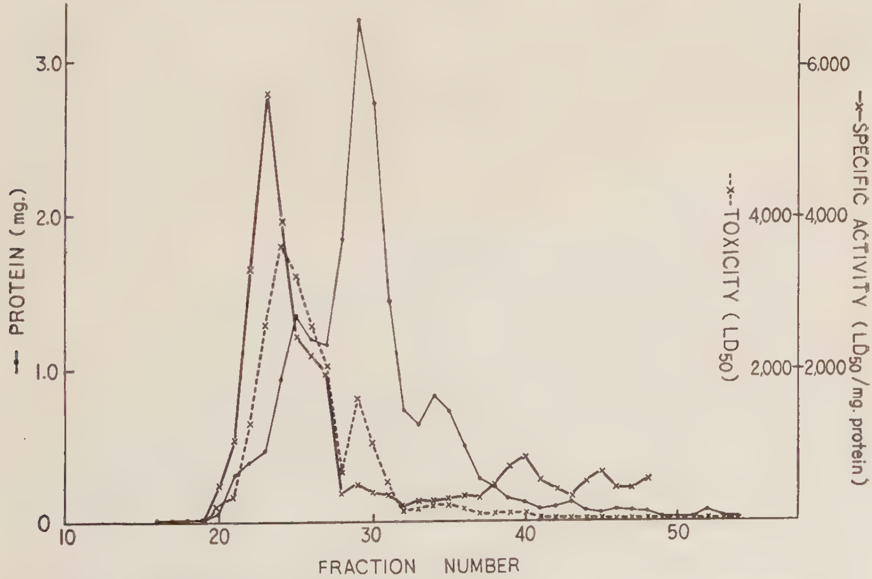


FIG. 6. Distribution of toxicities; LD$_{50}$ (--×--) and the specific activities (—×—) per mg. of protein in the same electrophoretic run as in Fig. 1.

mine their activities in each fraction. Because the veronal-HCl buffer will cause profound turbidity during the measurement of hyaluronidase activity by turbidity reducing method and make it impossible to determine the enzyme activity, zone electrophoresis was performed by using 0.1 *M* phosphate buffer, pH 7.6, instead of veronal-HCl buffer for the survey of the distribution of hyaluronidase activity in each fraction. Hyaluronidase activity was distributed over fractions No. 32 to 47 and constituted a high peak on the latter part of protein fraction which corresponds to the 3rd protein fraction obtained with veronal buffer (Fig. 5). However, it separated into two peaks of almost the same magnitude when expressed by specific activity. The enzyme activity recovery was 99.6 per cent.

The distribution of toxic principles in the venom was represented by LD_{50} in Fig. 6 with a recovery of 53.3 per cent. As Fig. 6 shows, although there are some toxic principles in the main (2nd) protein fraction, representing 18.3 per cent of the recovered toxicity, the majority of the toxicity, which represents approximately 74.3 per cent was concentrated in the 1st protein fraction.

Table I shows the increment in specific activities of enzymes and of toxicities by zone electrophoresis.

DISCUSSION

The electrophoretic patterns of Banded krait venom in veronal-HCl buffer, pH 8.6, ionic strength 0.1 and in phosphate buffer, pH 7.6, ionic strength 0.1, were quite different. In veronal buffer the venom migrated toward the cathode and separated very distinctly into one main protein fraction and two minor fractions. However, in phosphate buffer the venom revealed only one peak on the cathodic side.

According to Taborda *et al.* (*9*) the average phosphodiesterase content in venoms of various species showed a significant parallelism with their toxicity. Zeller (*10*) considered that 5′-nucleotidase and other phosphomonoesterase together with ATPase are probably responsible for the production of fatal shock. In the present experiment, however, phosphodiesterase, 5′-nucleotidase, ATPase, and DPN-pyrophosphatase activities of Banded krait venom are so feebly appreciated that only phosphomonoesterase which shows highest activity among the six kinds of Formosan snake venoms, and which would come into question, could be separated distinctly from the venom toxicity.

In agreement with Slotta (*11*) and Yang's finding (*1–4*), Banded krait venom gave an electrophoretically single fraction of phosphatidase A which just coincided with the 3rd protein fraction and was apparently separated from venom toxicity. L-Amino acid oxidase and cholinesterase, which display the highest activity among the Formosan snake venoms (Table III), were also separated from the venom toxicity and were revealed as an electrophoretically single fraction, just coinciding with the 3rd protein fraction. Since proteinase activity in the venom is so feeble, it would not be a cause of toxicity. Although hyaluronidase activity of Banded krait venom is highest among the six kinds of Formosan snake venoms studied (Table III), it was separated from toxicity by zone electrophoresis. Chiu (7) has also separated the hyaluronidase activity of cobra venom from its toxicity completely by ammonium sulfate fractionation.

Therefore, it is apparent, as we have reported, that none of the enzyme activities studied are toxicologically of prime importance (Fig. 7).

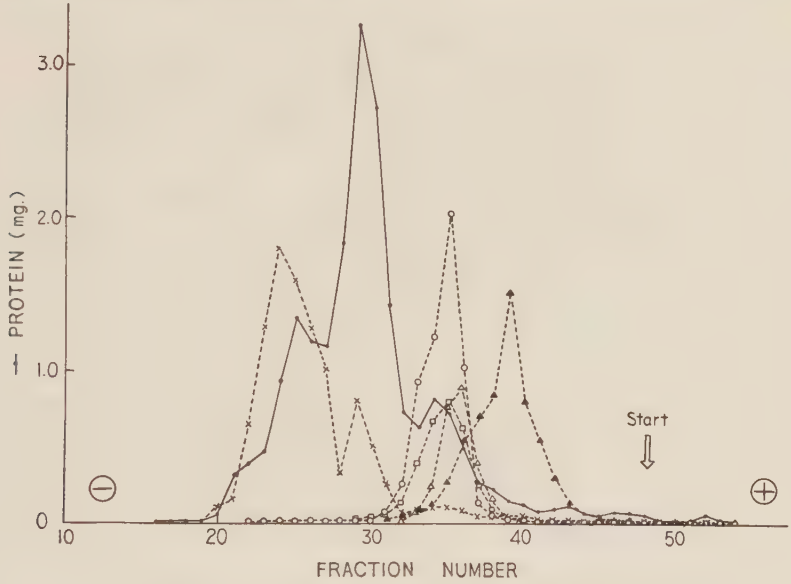


Fig. 7. Summary of the distribution of protein (—●—), phosphomonoesterase (—□—), phosphatidase A (—○—), cholinesterase (—▲—), L-amino acid oxidase (—△—), and toxicities (—×—) in the starch column electrophoresis of Banded krait venom.

SUMMARY

1. Banded krait (*Bungarus multicinctus*) venom was fractionated by zone electrophoresis into three fractions: one main protein fraction (2nd) with two minor (1st and 3rd) protein fractions on both sides of it.

2. Although some toxic principles were detected in the 2nd protein fraction, the majority of the toxicity, representing 74.3 per cent of the recovered toxicity, was concentrated in the 1st protein fraction.

3. All of the enzymes studied: phosphomonoesterase, phosphatidase A, cholinesterase, L-amino acid oxidase, and hyaluronidase activities were separated from the venom toxicity very distinctly, and were revealed as an electrophoretically single fraction just coinciding with the 3rd protein fraction.

4. Phosphodiesterase, 5′-nucleotidase, ATPase, DPN-pyrophosphatase,

and proteinase activities in Banded krait venom were so feeble, hence they would not be a cause of venom toxicity.

5. It was definitely proved that the venom toxicity has nothing to do with any of enzyme activities studied, confirming our previous work.

The authors are gratefully indebted to Prof. R. Hirohata, chief of the Laboratory of Protein Chemistry, Yamaguchi Medical School, Ube, for his continued interest and encouragement during the course of this study.

REFERENCES

(*1*) Yang, C. C., Chen, C. J., and Su, C. C., *J. Biochem.*, **46**, 1201 (1959)
(*2*) Yang, C. C.. Su, C. C., and Chen, C. J., *J. Biochem.*, **46**, 1209 (1959)
(*3*) Chen, C. J., *Yamaguti-igaku*, **8**, 575, 582 (1959)
(*4*) Chiu, W. C., *Yamaguti-igaku*, **9**, 1355 (1960)
(*5*) Yang, C. C., Chiu, W. C., and Kao, K. C., to be published.
(*6*) Su, C. C., *Yamaguti-igaku*, **8**, 862, 868 (1959)
(*7*) Chiu, W. C., *Yamaguti-igaku*, **9**, 1361 (1960)
(*8*) Yang, S. C., *Yamaguti-igaku*, **9**, 862 (1960)
(*9*) Taborda, A. R., Taborda, L. C., Williams, J. N., J., and Elvehjem, C. A. *J. Biol. Chem.*, **194**, 227 (1952)
(*10*) Zeller, E. A., "The Enzymes," Academic Press Inc., New York, Vol. **1**, 986 (1951)
(*11*) Slotta, K., and Borchert, P., *Mem. Inst. Butantan*, **26**, 297 (1954)
(*12*) Folin, O., and Ciocalteu, V., *J. Biol. Chem.*, **73**, 627 (1927)

The Journal of Biochemistry, Vol. 48, No. 5, 1960

AN ATTEMPT TO DEMONSTRATE THE SEPARATION OF F-ACTIN FROM MYOSIN B BY THE ACTIN OF ATP

By HARUHIKO NODA AND KOSÇAK MARUYAMA

(From the Department of Biophysics and Biochemistry, Faculty of Science and the Biological Institute, College of General Education, University of Tokyo, Tokyo)

(Received for publication, June 16, 1960)

Since the pioneering work of Szent-Györgyi's school (*1*), it has generally been accepted that myosin B is a complex of actin and myosin, *i.e.* actomyosin, and that adenosinetriphosphate (ATP) causes myosin B to dissociate into f-actin and myosin. Weber (*2*) successfully isolated myosin from myosin B in 0.6 M KCl solution under the influence of ATP by way of ultracentrifugation. Her demonstration of the separation of f-actin was not so clear, and this lead some workers (*3, 4*) to doubt the actual participation of f-actin in the formation of myosin B molecules. Several investigators (*5–7*) succeeded in demonstrating the presence of actin as a chemical component in myosin B, but a final demonstration of the separation of f-action from myosin B by the influence of ATP has not yet been made.

In the course of the study of the myosin B-ATP system with the flow birefringence technique (*8*), most of the results were explained by the hypothesis that ATP causes myosin B to dissociate into f-action and myosin. Therefore attention has been focussed on the separation of f-actin from myosin B under the influence of ATP. The ultracentrifugation experiment of Weber (*2*) was repeated, and the pellet was partly solubilized. The protein solution thus obtained and its reaction with myosin and ATP was examined with the flow birefringence technique, and the results indicated that this protein was f-action. From the standpoint of amino acid analysis also, this protein was identified as actin.

EXPERIMENTAL METHODS

Twenty-four hour myosion B was prepared from rabbit skeletal muscle as described previously (*8*). The purification method was slightly modified, *i.e.*, the precipitation of myosin B was effected at 0.2 M KCl, in order to minimize the contamination by myosin A. The myosin B solution obtained was clarified by centrifuging at 70,000×g for an hour at a protein concentration of 1 to 1.5 per cent. The procedure to separate actin from the clarified myosin B solution under the influence of ATP will be described in the results section.

Myosin A, or pure myosin, was kindly supplied by Drs. S. and F. Ebashi, Faculty of Medicine, University of Tokyo. Highly purified actin prepared from rabbit muscle according to the method of Straub (*9*) was kindly furnished by Drs. F. Ôosawa and S. Asakura of Nagoya University.

The technique of flow birefringence used was described in the previous report (*8*). The protein concentration was determined by the Kjeldahl method and in some cases by the Biuret reaction. The scattered light intensity at right angle was determined with A.K.A. nephelometer manufactured by Kotaki Co. of Tokyo.

RESULTS

Fractionation of Myosin B under the Influence of ATP—Weber (*2*) first separated myosin from myosin B in 0.6 *M* KCl solution by centrifuging at 100,000×*g* for 3 hours in the presence of ATP. We repeated the same experiment, and observed that myosin remained in the supernatant portion (*8*) thus confirming her conclusion. Then an effort was concentrated upon the solubilization of the pellet formed in such a centrifugation. In a few preliminary trials, only a trace of protein could be extracted by suspending the pellet in a solution of 10^{-4} *M* ATP or 0.6 *M* KCl as was already stated by Weber (*2*). It was then suspected that a thorough grinding of the pellet was necessary for the solubilization. After repeated homogenization by means of a glass rod apparatus of the Potter type, however, only a typical myosin B solution was obtained. This was assumed to be due to the presence in the pellet of myosin A which readily recombined with the extracted f-actin.

Therefore attempts were made to remove myosin A from the pellet. The pellet was first homogenized in 0.6 *M* KCl containing 1×10^{-3} *M* ATP and immediately centrifuged. This washing solution contained myosin. The washing procedure was repeated, and the second washing solution contained myosin and f-action. The washed ground pellet was again homogenized in 0.6 *M* KCl and the total mixture was left standing in a refrigerator over night. The final extract thus obtained was tested with various physico-chemical methods, and the results showed that the fibrous protein in this extract was f-actin and not myosin B. Detailled characterization

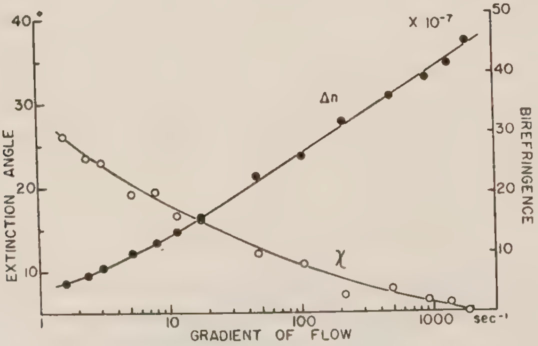


FIG. 1. Flow birefringence properties for f-actin extracted from myosin B with 0.6 *M* KCl solution.

of this protein will be given in the following. The yield of f-actin was low; about 3 to 5 per cent of the total protein of the initial myosin B solution.

In another experiment, the well washed pellet was homogenized in 1×10^{-4} *M* ATP and dialyzed against a solution of 1×10^{-4} *M* ATP, ascorbate and $NaHCO_3$, which was supposed to depolymerize f-actin into g-actin (*9*). After dialysis for 2 days, the supernatant solution was separated by centrifugation. The supernatant solution did not show flow birefringence. When the amino acid analysis of the supernatant solution was performed, the amino acid composition was found to be the actin type.

In a third experiment, the washed pellet was extracted with pure myosin solution in 0.6 *M* KCl. The supernatant became very viscous, and by sedimentation and flow birefringence it was found to be a typical solution of myosin B.

These experiments were performed on four independent preparations of myosin B, and the results were all the same. The amount of protein distributed in various fractions in a typical case is shown in Table I.

TABLE I

Fractionation of Myosin B under the Influence of ATP

	Fractions	Main components	Amount of protein derived from 10 ml. of the original myosin B solution. (mg.)
1.	Original solution of myosin B.		58
2.	Centrifugation at $100{,}000\times g$ for 3 hours.		
a.	Total supernate.	Myosin + f-actin.	42
b.	Upper 3/10 of the supernate.	Myosin.	7
c.	Lower 7/10 of the supernate.	Myosin + f-actin.	34
d.	Pellet	—	20
3.	Extraction of the pellet with 0.6 *M* KCl.		
a.	1st ATP-KCl washing solution.	Myosin.	3.2
b.	2nd ATP-KCl washing solution.	Myosin + f-actin.	1.4
c.	KCl extract.	F-actin.	3.6
d.	Residue.	—	6.3
4.	Extraction of the pellet with myosin A solution.		
a.	Myosin-KCl extract.	Myosin B.	14
b.	Residue.	—	8.4
c.	Myosin A for extraction.	Myosin A.	10.5
5.	Depolymerization of the pellet.		
a.	Depolymerized supernate.	G-actin.	4.9
b.	Residue.	—	6.3

For details, see the text.

In one case the washed pellet was extracted with 0.1 *M* KCl, pH 7.0, while stirring the solution with a magnatic stirrer over night at 2–3°. The extracted protein concentration was about 3 per cent of the initial protein concentration, and the solution showed marked birefringence of flow (Fig. 2). When the ionic strength of this solution was lowered by dialysis, the flow birefringence vanished.

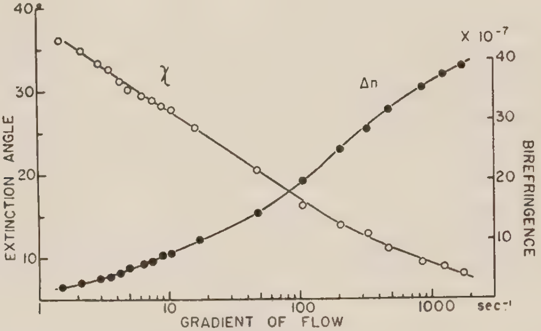


FIG. 2. Flow birefringence properties for f-actin extracted from myosin B with 0.1 *M* KCl solution.

Flow Birefringence Properties of F-actin from Myosin B—The 0.6 *M* KCl extract of the pellet, which contained f-actin, showed clear and strong flow birefringence even at a very low protein concentration of 0.01 per cent. The relation of the extinction angle (χ) and the birefringence (Δn) to the velocity gradient of flow (G) is shown in Fig. 1. When this relation is compared to that of myosin B, χ at low G is lower and Δn is much larger. ATP had almost no effect on Δn of the solution, and even when there was a change, the increase or decrease in Δn caused by ATP addition was not more than 10 per cent. The amount of Δn at high G was around 10^{-5} per 0.1 per cent protein concentration, and this agreed well with the results obtained with usual f-actin (*10*).

The solution of the f-actin from myosin B in 0.6 *M* KCl showed some signs of instability; dilution caused decrease in the specific birefringence, and when the solution was left standing at 0° over night, the birefringence decreased by a considerable fraction. On the contrary, the 0.1 *M* KCl extract of the pellet was more stable. The properties shown in Fig. 2 were preserved for three days while the solution was kept standing at 0°. The χ-G relationship for this extract was similar to that for myosin B, but the specific birefringence for this solution was far greater than that for myosin B.

The molecular length calculated for f-actin from myosin B ranged from 1 to 3 micra (Table II) and was very similar to that of myosin B (*8*).

Reconstituted Actomyosin—When the f-actin from myosin B in 0.6 *M* KCl was mixed with myosin, a complex was formed which was very similar to myosin B. Superprecipitation clearly took place when the ionic strength was lowered to 0.1 M KCl and ATP was added. Any solution of the f-actin or myosin alone showed no such response to ATP.

TABLE II

Length of F-actin Molecules from Myosin B.

F-actin in 0.6 M KCl.

G	Rotary diffusion constant	Length[1]
10	1.0 (sec.$^{-1}$)	2.6 (μ)
100	2.4	2.0
1000	11	1.2

F-actin in 0.1 M KCl.

G	Rotary diffusion constant	Length
0	0.45	3.4
10	2.1	2.1
100	5.0	1.6
1000	19	1.0

1) The apparent length was calculated by assuming the molecules to be prolate ellipsoids.

TABLE III

The Intensity of Scattered Light by Reconstituted Actomyosin

	Relative intensity of scattered light at 90°
Myosin.	18 } 32
F-actin from myosin B.	14 }
Reconstituted actomyosin.	48
Reconstituted actomyosin+ATP.	30
Myosin B.	65
Myosin B+ATP.	26

At 20°, pH 7.0, and the final concentration of ATP was 1 mM when used.

The addition of ATP to the solution of the complex of myosin and f-actin in 0.6 *M* KCl caused a change of light scattering by the solution very similar to the case with myosin B (Table III). The amount of scattered light increased upon complex formation, and with the addition of ATP reverted to the sum of initial values. The viscosity of the solution of the complex was also reduced by ATP, and the ATP sensitivity (*11*) was 50 per cent at 20°. The complex of f-actin and myosin showed flow birefringence properties very similar to those of myosin B, and the change caused by ATP addition was also very similar as are shown in Figs. 3 and 4.

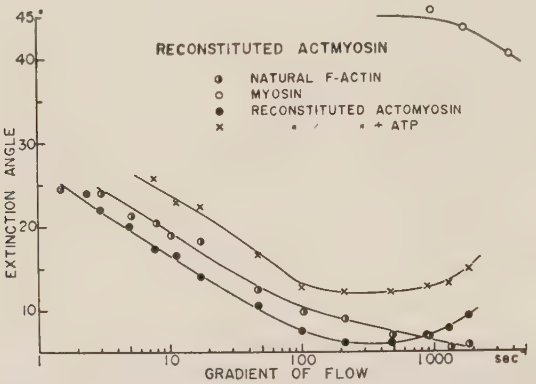


Fig. 3. Extinction angle for reconstituted actomyosion and its change with the addition of ATP.

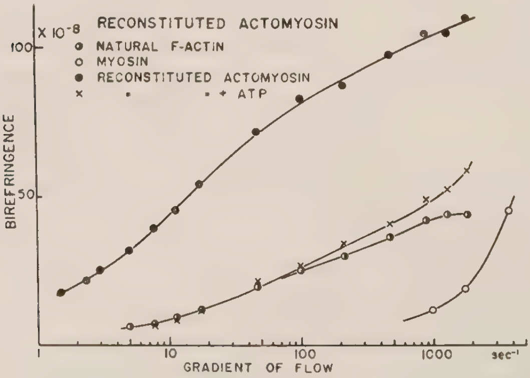


Fig. 4. Birefringence for recontituted actomyosin and its change with the addition of ATP.

Amino Acid Analysis—It was pointed out by Kominz, Hough, Symonds and Laki (*12*) that one of the obvious differences for actin and myosin was the difference in their amino acid composition. Upon examination of their results of total amino acid analysis, it was found that the difference in the content of proline was the most prominent between actin and myosin. On the other hand, the content cf arginine was almost the same for both proteins. Moreover, for the analysis of proline and arginine, there are specific colorimetric methods for each of them. Therefore, the ratio of the proline content to that of arginine was chosen as an indicator. According to the result of Kominz *et al.* (*12*), the ratio Pro/Arg is 0.54 for myosin and 1.15 for actin.

In preliminary experiments in which pure myosin and pure actin were analysed, the results obtained were in excellent agreement with the above mentioned ratios with no deviation of the value greater than 5 per cent for about 10 independent determinations on different batches of protein preparations. The results for various protein fractions obtained in the present experiment are shown in Table IV.

TABLE IV

Ratio of Proline to Arginine in Muscle Protein

		Pro./Arg.
1.	Myosin.	
	Pure myosin A.	0.54
	Myosin A prepared according to the method of A. Weber.	0.54
2.	Actin.	
	Actin prepared directly from muscle by the method of Straub.	1.15
	Total pellet of centrifugation of myosin B with ATP.	0.96
	0.6 *M* KCl extract of tbe pellet.	0.96
	The residue of the pellet after extraction with 0.6 *M* KCl.	1.25
	Solubilized portion of the pellet with ATP containing water.	1.11
	G-actin obtained from the pellet of myosin B and PP.	1.17
3.	Myosin B.	
	24 hour myosin B.	0.74
	Clarified myosin B. (at 60,000×g for 3 hours).	0.74

Total amino acid analyses were also performed on several preparations, and the results coincided well with those of Kominz *et al.* (*12*).

DISCUSSION

In the present experiment, it was made clear that when extracted with a suitable medium, the sedimentation pellet formed in 0.6 *M* KCl from myosin B under the influence of ATP, yielded a solution of protein of long fibrous molecules. The length of such molecules was close to that of myosin

B (*8*), but this protein had no ATPase activity or suffered no change in the physico-chemical properties with the addition of ATP. When this extracted protein was combined with myosin, the resulting complex showed the properties of myosin B (Figs. 3 and 4). Therefore, it was concluded that this extracted protein was the f-actin on the ground of the hypothesis that ATP causes myosin B to dissociate into f-actin and myosin.

One of the important properties of actin is its reversible association and dissociation caused by the change in the ionic strength of the medium. When the high speed sedimentation pellet was dialysed against water which contained low concentration of ATP and ascorbic acid only, a protein of relatively low molecular weight came into solution. This protein showed no flow birefringence in the shear rate range of up to 4000 sec.^{-1}, and its amino acid composition was that of actin. However, upon addition of KCl or $MgCl_2$, this protein failed to show any sign of f-actin formation, except for just one case in which there was an increase in viscosity suggesting the f-actin formation. Although no explanation for this loss of polymerizability was attained so far, it was not unexpected from the fact that even in the course of purification of actin by the method of Mommaerts (*9*), a considerable portion of actin is inactivated and loses the ability to form f-actin. Moreover, since the concentration of the extraction solution of the f-actin was very low, it may be lower than the critical concentration (*10*) under which even active g-actin fails to polymerize.

The f-actin from myosin B formed a complex with myosin, and the properties of the complex as described in the results section indicated that this complex was a reconstituted actomyosin. There were, however, indications that the affinity of the f-actin to form actomyosin with myosin was damaged to some extent during the preparative procedures. The change in the intensity of scattered light caused by ATP was smaller than in the case of myosin B, *i.e.* the natural actomyosin. The ATP sensitivity of the viscosity of the reconstituted actomyosin was only 50 per cent while that for myosin B is usually 100 per cent. When examined with the technique of flow birefringence, the amount of birefringence for reconstituted actomyosin was not as high as expected from total alignment of myosin molecules along f-actin molecules, and ATP caused more marked increase in χ for the reconstituted actomyosin than for myosin B. All these results seem to indicate that the reconstituted actomyosin was more ready to dissociate in comparison with myosin B. Part of such characteristics may be ascribed to the instability of the f-actin from myosin B in 0.6 M KCl.

As is seen in Table IV, for the f-actin, the ratio of Pro/Arg was 0.96, which is the same as that for the total pellet formed by centrifuging myosin B with ATP. The insoluble residue of the pellet after extraction with 0.6 M KCl was almost a pure actin with the ratio of 1.25. These results of amino acid analyses suggest that pure f-actin molecules become completely insoluble when it is packed as a pellet by high speed centrifugation, and in order to be extracted by 0.6 M KCl, the f-actin should have some myosin around f-actin molecules. However, it should be pointed out that the solution of f-actin from myosin B showed no ATPase activity. It seems that myosin contained in the extracted f-actin is combined with actin in a very different manner from that in actomyosin, either natural or reconstituted.

The values of Pro/Arg for myosin B before and after clarification centrifugation at 60,000×g for 3 hours were both 0.74, indicating such centrifugation has nothing to separate any protein component of amino acid compositson different from that of myosin B. From the value of 0.74 for Pro/Arg, the ratio of myosin to actin in the formation of myosin B is calculated to be about 2:1, which is somewhat different from the commonly accepted ratio of 4:1 (*1*). The amount recovered as f-actin was 3 to 5 per cent of the original myosin B, thus the yield was very low when the above ratio is taken into consideration. However, this is not so surprising; most of f-actin became packed into the insoluble pellet during centrifugation. When synthetic actomyosin was centrifuged with ATP, Gergely and Martonosi* observed that f-actin pellet became insoluble.

It is verified, by the experiments described in the above, that f-actin separates from myosin B under the influence of ATP. The present results strongly support the current idea (*1*, *11*) that myosin B is a natural actomyosin. As regards its interaction with ATP, however, a complete dissociation into myosin and f-actin is rather improbable. Even in the presence of ATP, the interaction between actin and myosin seems to remain to some extent. In the flow birefringence experiments on myosin B (*8*), some phenomena were observed which may not be explained by the dissociation theory. In this connection, it is notable that Morales' (*3*) and Tonomura's (*13*) school proposed that part of myosin B molecules extend in the presence of ATP, in order to interprete their results of light scattering experiments.

SUMMARY

A fibrous protein was extracted from the pellet formed by centrifuging the solution of myosin B in the presence of ATP. This protein had properties which were to be expected for the f-actin component separated from myosin B by the action of ATP. The length of the molecule estimated from the measurement of flow birefringence was about 1 to 3 micra which agreed well with that for myosin B. The amino acid composition of this protein was similar to actin obtained directly from muscle by the method of Straub.

When the f-actin from myosin B was combined with myosin A, reconstituted actomyosin was formed. The properties of the reconstituted actomyosin and its reaction with ATP were quite similar to those for myosin B from the viewpoints of flow birefringence, light scattering and superprecipitation.

---

* Gergely, J., and Martonosi, A., personal communication.

REFERENCES

(*1*) Szent-Györgyi, A., *Acta Physiol. Scand.*, **9**, Suppl. 25 (1945)
(*2*) Weber, A., *Biochim. et Biophys. Acta*, **19**, 345 (1956)
(*3*) Von Hippel, P. H., Gellert, M. F., and Morales, M. F., 'Conference on the Chemistry of Muscular Contraction', Tokyo (1958)
(*4*) Gellert, M. F., von Hippel, P. H., Schachman, H. K., and Morales, M. F., *J. Am. Chem. Soc.*, **81**, 1384 (1959)
(*5*) Szent-Györgyi, A. G., *J. Biol. Chem.*, **192**, 361 (1951)
(*6*) Barany, M., Barany, K., and Guba, F., *Nature*, **179**, 818 (1957)
(*7*) Gergely, J., *Ann. New York Acad. Sci.*, **72**, 538 (1959)
(*8*) Noda, H., and Maruyama, K., *Biochim. et Biophys. Acta*, **30**, 598 (1958)
(*9*) Mommaerts, W. F. H. M., *J. Biol. Chem.*, **198**, 445 and 469 (1952)
(*10*) Oosawa, F., Asakura, S., Hotta, K., Imai, N., and Oi, T., *J. Polymer Sci.*, **37**, 323 (1959)
(*11*) Portzehl, H., Schramm, G., and Weber, H. H., *Z. Naturforsch.*, **5b**, 61 (1950)
(*12*) Kominz, D. R., Hough, A., Symonds, P., and Laki, K., *Arch. Biochem. Biophys.*, **50**, 148 (1954)
(*13*) Nihei T., and Tonomura, Y., *J. Biochem.*, **46**, 1355 (1959)

The Journal of Biochemistry, Vol. 48, No. 5, 1960

ENZYMATIC STUDIES ON PYRIDOXINE METABOLISM

IV. A PYRIDOXINE DEHYDROGENASE FROM BAKER'S YEAST*

By YOSHIMASA MORINO and YUKIYA SAKAMOTO

(*From the Biochemical Department, Medical School, Osaka University, Osaka*)

(Received for publication, June 20, 1960)

Recently we have reported that rabbit liver extracts contain two distinct enzymes or enzyme systems, the one catalyzing the oxidation of pyridoxine phosphate to pyridoxal phosphate and the other the conversion of pyridoxine to pyridoxic acid in the presence of an aldehyde oxidase preparation (*1*). Subsequently, pyridoxine phosphate oxidase was purified from rabbit liver extracts and was demonstrated to be a flavin enzyme (*2*). The characterization of the pyridoxine oxidizing enzyme system, however, remained unknown. During the course of this study, pyridoxal was found to decrease when incubated with an enzyme preparation from baker's yeast in the presence of TPNH (*3*).

This paper reports on the purification and properties of an enzyme which is involved in the interconversion of pyridoxine and pyridoxal. This enzyme will be designated as pyridoxine dehydrogenase. The role of this enzyme in the metabolism of pyridoxine in baker's yeast will also be discussed.

MATERIALS AND METHODS

Pyridoxine hydrochloride was kindly given by Dr. Matsukawa of Takeda Pharmaceutical Company, Osaka. 5-Deoxypyridoxal and isopyridoxal were kindly given by Dr. E. E. Snell. TPN was purchased from Sigma Chemical Company. Pressed cakes of baker's yeast were obtained from Oriental Yeast Company, Osaka.

Glucose-6-phosphate dehydrogenase was prepared from baker's yeast by a modification of the method described by Kornberg (*4*).

Pyridoxal was determined using 2,4-dinitrophenylhydrazine**. A typical determination procedure was as follows. The incubation mixture was deproteinized with trichloroacetic acid (5 per cent final concentration) and the supernatant solution after centrifugation was made up to 4.0 ml. with deionized water. Then 0.5 ml. of 2 per cent 2, 4-dinitrophenylhydrazine (in $10\,N$ H_2SO_4) was added. After standing for 30 minutes at 37°, the solution was shaken with 5 ml. of a mixture of ethyl acetate and diethylketone (9: 1) and the solvent layer was removed. This extraction procedure was repeated until the excess 2,4-dinitrophenylhydrazine had been completely extracted from the aqueous layer. The optical density of the hydrazone-containing aqueous layer was measured at 415 mμ. A standard curve was made with pyridoxal. 5-Deoxypyridoxal was measured in a similar way. The optical density of isopyridoxal-hydrazone was read at 386 mμ.

---

* This work was presented at the 7th Kinki Local Meeting of the Japanese Biochemical Society (Osaka) in May, 1960.

** We owe this determination method to a kind suggestion by Dr. H. Wada.

The identification of pyridoxine, pyridoxal, isopyridoxal 5-deoxypyridoxine and 5-deoxypyridoxal was carried out by ascending paper chromatography using Toyo Roshi filter paper No. 51 in the solvent system (acetone, 35: *tert*-butanol, 40: diethylamine, 5: water, 20) (*5*).

RESULTS

Purification of the Enzyme—Air-dried yeast powder was autolyzed by shaking with three volumes of 0.1 *M* sodium bicarbonate for 3 hours at 37°. After cooling to about 10°, the autolyzate was diluted with 3/4 volume of deionized water. Subsequent operations were carried out below 2°. The mixture was then centrifuged at 15,000 × *g* for 20 minutes. To each 100 ml. of the supernatant were added 35.1 g. of solid ammonium sulfate. The precipitate was centrifuged off. An additional 7 g. of solid ammonium sulfate were added to the supernatant. The resulting precipitate was dissolved in deionized water and dialyzed against 0.001 *M* potassium phosphate buffer, pH 7.4, containing 0.001 *M* glutathione. To the dialyzate was added 0.35 g. (dry weight) of alumina gel Cγ per g. of protein. After adjusting the pH to 6.0 with 1 *M* acetate buffer of pH 4.9, the preparation was allowed to stand for 15 minutes in the cold with occasional stirring. The gel was collected by brief centrifugation. The supernatant had no enzyme activity. The gel was eluted by stirring it with 30 ml. of 0.1 *M* potassium phosphate buffer, pH 7.4, and then the mixture was allowed to stand for

TABLE I

Purification of Enzyme

Purification step	Total volume	Specific[1] activity	Yield
	ml.		per cent
Crude extract from 50 mg. of air-dried baker's yeast	258	1.7	100
Dialyzed ammonium sulfate fraction (55%~66%)	42	22.5	43
Gel eluate	20	125	36
Second ammonium sulfate fraction, dialyzed and lyophilized	—	140	—

1) Specific activity was defined as mμ mole of pyridoxal formed per mg. of protein in 10 minutes at 37°.

The incubation mixture contained, in a volume of 1.0 ml., 10 μM of pyridoxine, 1.0 μM of TPN, an appropriate amount of enzyme preparation and 40 μM of sodium carbonate bicarbonate buffer, pH 9.0. Incubated for 5 minutes at 37°.

15 minutes. The supernatant after centrifugation contained the major portion of the pyridoxine dehydrogenase. This eluate was subjected to a further fractionation with ammonium sulfate. The portion precipitating between 0.6 and 0.7 saturation with ammonium sulfate was collected and dissolved in a minimal volume of deionized water. The resulting enzyme solution was dialyzed against 0.005 *M* potassium phosphate buffer, pH 7.4. The dialyzate was lyophilized. The lyophilized preparation was stable for 2 months when kept dry and cold. In an aqueous solution the enzyme lost about 50 per cent activity when heated at 55° for 2 minutes and, was completely inactivated by heating at 60° for 2 minutes.

Results of the purification procedure were shown in Table I.

Identification of Reaction Product—The oxidation product was inferred to be pyridoxal since the complete reaction mixture became yellow during incubation. The difference in the absorption spectrum of the complete reaction mixture and a reaction mixture lacking TPN also corresponded to the typical spectrum of pyridoxal. Furthermore, the reaction product was identified by paper chromatography. The reaction mixture containing, in a volume of 1.0 ml., 10 μ moles of pyridoxine, 1.5 μ moles of TPN, 2.0 mg. of purified enzyme preparation and 40 μ moles of sodium carbonate bicarbonate buffer, pH 9.0, was incubated at 37°. After 20 minutes incubation, 4 ml. of 99 per cent of ethanol were added. After centrifugation the supernatant was evaporated to about 0.5 ml. under reduced pressure. An aliquot of the concentrate was applied to filter paper (Toyo Roshi No. 51) in the solvent system described by Snell. A chromatogram of the complete reaction mixture revealed two spots corresponding to pyridoxine and pyridoxal.

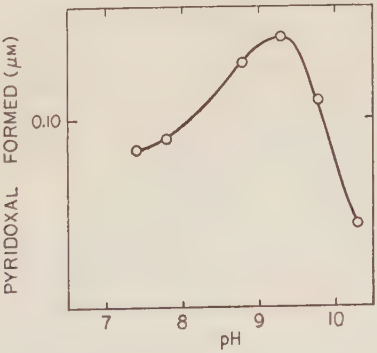


Fig. 1. Effect of pH on the reaction rate. The reaction mixture contained, in a volume of 1.0 ml., 10 μM of pyridoxine, 1.2 μM of TPN, 1.1 mg. of purified pyridoxine dehydrogenase and 50 μM of potassium phosphate buffer or sodium carbonate buffer of the indicated pH. Incubated for 10 minutes at 37°.

Effect of pH on the Reaction Rate—The pH activity curve of pyridoxine dehydrogenase is shown in Fig. 1. A sharp decline in activity was observed above pH 9.5.

Effect of Pyridoxine Concentration on the Reaction Rate—The effect of the pyridoxine concentration on the rate of pyridoxal formation is shown in Fig. 2. A maximal velocity was obtained with 0.01 *M* pyridoxine. The Km for pyridoxine was calculated from double reciprocal plots as $1.6\times10^{-3}M$.

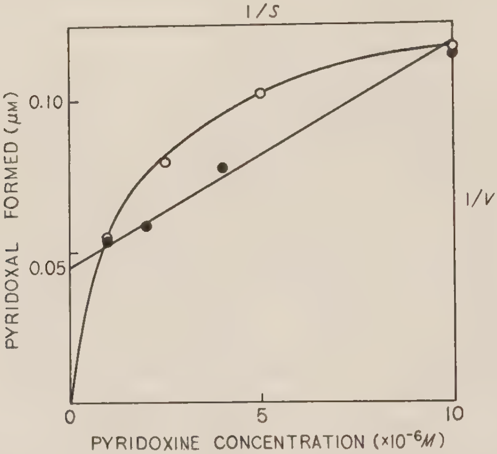


Fig. 2. Effect of substrate concentration on the reaction rate.

The incubation mixture contained, in a volume of 1.0 ml., the incubated amount of pyridoxine, 1.2 μM of TPN, 1.0 mg. of pyridoxine dehydrogenase and 40 μM of sodium carbanate buffer, pH 9.0. Incubated for 10 minutes at 37°.

Absolute Requirement for TPN as Electron Acceptor—In the previous report (*3*), it was demonstrated that pyridoxal decreased only when incubated with a yeast enzyme preparation in the presence of a TPNH regenerating system. Table II shows more detailed data on the cofactor requirements. In a preliminary experiment, it was found that the amount of pyridoxal formed more than accounted for the TPN added. To prevent the turnover of TPN (probably due to a contamination by TPNH-oxidase), the effect of the TPN concentration on the reaction rate was studied under anaerobic conditions (Fig. 3). From the data a Km for the coenzyme was determined as $6.0\times10^{-4}M$.

Time Course of the Reaction—As can be seen from Fig. 4, the reaction proceeded in a nearly linear fashion for the first 5 minutes and reached an equilibrium after 60 minutes. As expected the amount of pyridoxal present at equilibrium was less at pH 7.6 than that at pH 9.0.

Reversibility of the Reaction—Some of the relative data was shown in Table II. By analogy to other pyridine nucleotide requiring enzyme, it was expected that the backward reaction, *i.e.*, reduction of pyridoxal would

TABLE II

Coenzyme Requirement

a) Conversion of pyridoxine to pyridoxal.

The complete incubation mixture contained, in a volume of 0.6 ml., 10 μM of pyridoxine, 0.8 μM of TPN, 6 μM of reduced glutathione, 0.6 mg. of pyridoxine dehydrogenase and 40 μM of sodium carbonate buffer, pH 9.0. Incubated for 10 minutes at 37°.

Condition	Pyridoxal formed
Complete system	0.21 μM
−TPN	0.00
−Enzyme	0.00
−TPN, +DPN (10 μM)	0.00

b) Conversion of pyridoxal to pyridoxine.

The complete incubation mixture contained, in a volume of 0.6 ml., 0.5 μM of pyridoxal, 0.2 μM of TPN, 5 μM of glucose-6-phosphate, 0.4 mg. of glucose-6-phosphate dehydrogenase, 0.6 mg. of pyridoxine dehydrogenase and 40 μM of sodium carbonate buffer, pH 9.0. Incubated for 10 minutes at 37°.

Condition	Pyridoxal disappeared
Complete system	0.45 μM
−TPN	0.00
−glucose-6-phosphate	0.00
−TPN, glucose-6-phosphate, +DPNH (5 μM)	0.00

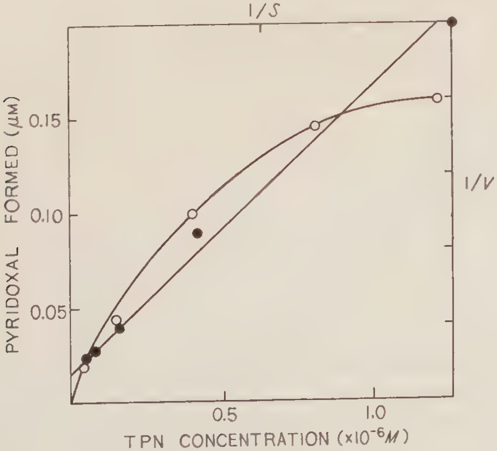


FIG. 3. Effect of TPN concentration on the reaction rate. The reaction mixture contained, in a volume of 1.0 ml., 10 μM of pyridoxine, the indicated amount of TPN, 1.2 mg. of pyridoxine dehydrogenase and 40 μM of sodium carbonate buffer pH 9.0. Incubated for 10 minutes at 37°.

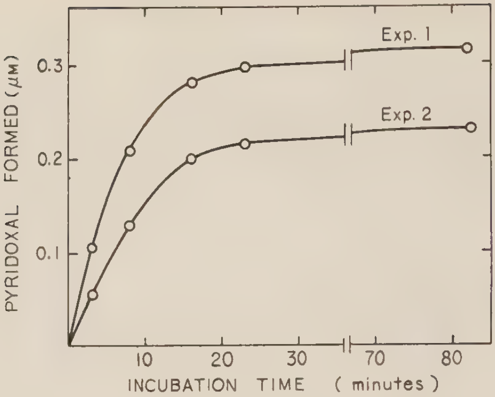


FIG. 4. Time course of the reaction.

Each tube contained, in a volume of 1.0 ml., 10 μM of pyridoxine, 2.0 μM of TPN, 1.9 mg. of pyridoxine dehydrogenase and 40 μM of sodium bicarbonate buffer, pH 9.0 (in the experiment 1), or 100 μM of potassium phosphate buffer, pH 7.6 (in the experiment 2). The incubation was carried out at 37° and stopped at the indicated time.

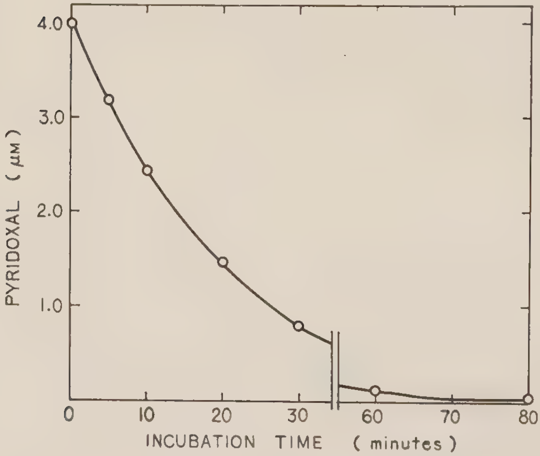


Fig. 5. Time course of pyridoxal reduction.

The incubation mixture contained, in a volume of 4.6 ml., 4 μM of pyridoxal, 0.4 μM of TPN, 20 μM of glucose-6-phosphate, 1.5 mg. of glucose-6-phosphate dehydrogenase, 4.2 mg. of pyridoxine dehydrogenase and 200 μM of sodium carbonate buffer, pH 9.0.

Incubation was carried out at 37° and 0.2 ml. aliquot was removed from the incubation mixture at the indicated time.

proceed more readily than the oxidation of pyridoxine. Indeed it was found that pyridoxal disappeared rapidly when incubated with the enzyme preparation in the presence of an excess of a TPNH regenerating system. The time course of pyridoxal reduction is shown in Fig. 5. Under these conditions, 1.0 μ mole pyridoxal was reduced in a linear fashion during the first 10 minutes. From the data in Fig. 4 and 5, it is highly probable the reaction would favor pyridoxine formation, although its exact equilibrium constant could not be measured*. The effect of the pyridoxal concentration on the rate of pyridoxal reduction is shown in Fig. 6. The Km of $4.4\times 10^{-5}M$ for pyridoxal was calculated from these data, much smaller than that for pyridoxine.

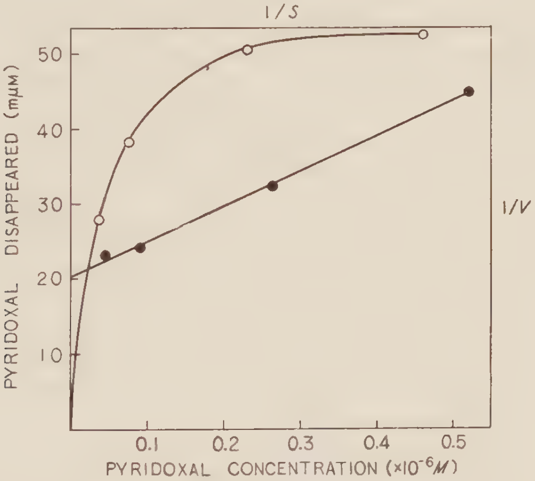


Fig. 6. Effect of pyridoxal concentration on the rate of pyridoxal reduction.

Each tube contained, in a volume of 0.5 ml., the indicated amount of pyridoxal, 0.2 μM of TPN, 5.0 μM of glucose-6-phosphate, 0.4 mg. of glucose-6-phosphate dehydrogenase, 0.8 mg. of pyridoxine dehydrogenase, 1.0 μM of glutathione and 40 μM of sodium carbonate buffer, pH 9.0, and was incubated for 2 minutes at 37°.

Substrate Specificity—The pyridoxine dehydrogenase was expected to be concerned specifically with interconversion of the hydroxymethyl- and formyl-groups at position 4 of the pyridine ring. In order to prove this, isopyridoxal, 4-deoxypyridoxine and 5-deoxypyridoxal were examined to see whether they were substrates for pyridoxine dehydrogenase. 5-Deoxypyridoxal was reduced in the presence of TPNH at a rate comparable to that of pyridoxal but the apparent affinity of the enzyme for it ($3.7\times 10^{-4}M$), was considerably lower than that for pyridoxal (Fig. 7). 5-Deoxypyridoxine which was prepared by reduction of 5-deoxypiridoxal with sodium borohydride was also oxidized to 5-deoxypyridoxal in the presece of TPNH and pyridoxine dehydrogenase. However, 4-deoxypyridoxine could not be oxidized by the enzyme nor did it inhibit the oxidation of pyridoxine. Pyridoxine phosphate was not oxidized under the same conditions.

---

* The apparent equilibrium constant was roughly calculated to be of the order of 10^{-10} from the data in Fig. 4.

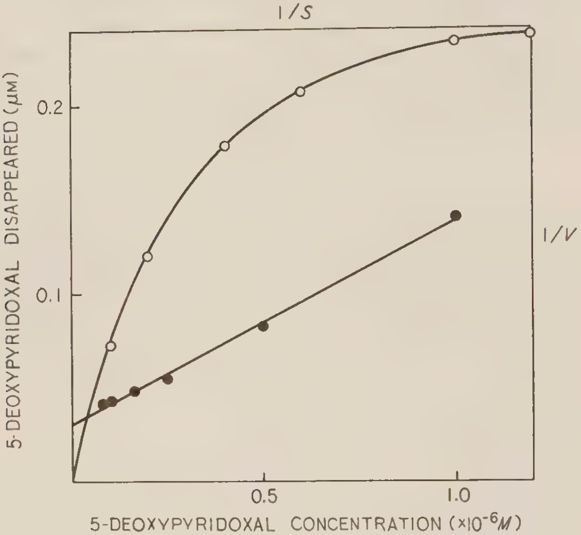


Fig. 7. Effect of 5-deoxypyridoxal concentration on the rate of 5-deoxypyridoxal reduction.

Incubation conditions were similar to those of Fig. 6 except that 5-deoxypyridoxal replaced pyridoxal and incubation was carried out for 3 minutes.

Activators and Inhibitors—Among the metal ions tested, $2.0\times10^{-5}M$ of Co^{++} enhanced the reaction rate about 90 per cent and, $2\times10^{-3}M$ of Mg^{++}, about 60 per cent. Mn^{++}, Ni^{++} and Fe^{+++} showed no appreciable effect at either concentration. Zn^{++} strongly inhibited the enzyme action. Metal binding agents such as α, α'-dipyridyl, EDTA* and *o*-phenanthroline somewhat stimulated the reaction rate at concentration of $1\times10^{-3}M$. This seems to be due to the removal of contaminating inhibitory heavy metal ions. These data were summarized in Table III.

Table IV shows data on the sulfhydryl character of the enzyme. p-CMB** caused 40 per cent inhibition at a concentration of $1\times10^{-5}M$ and 100 per cent at $5\times10^{-5}M$. When the apoenzyme was preincubated with TPN, however, inhibition by p-CMB was greately reduced. Pyridoxine had no such protecting action. The enzyme activity was stimulated by reduced

---

* The abbreviation used is: EDTA, ethylenediamine tetraacetic acid.

** The abbreviation used is: p-CMB, p-chloromercuribenzoic acid.

TABLE III

Effect of Metal Ions and Metal Chelating Agents on the Enzyme Activity

Each tube contained, in a volume of 1.0 ml., 10 μM of pyridoxine, 1.0 μM of TPN, 0.9 mg. of pyridoxine dehydrogenase, the indicated amount of each metal salt or chelating agent and 40 μM of sodium carbonate buffer, pH 9.0. Incubation was carried out for 5 minutes at 37°.

Addition	Concentration	Activity	% Activity
	M	mμM pyridoxal	
none		50	100
$CoCl_2 \cdot 6H_2O$	3.3×10^{-6}	53	106
	2.0×10^{-5}	94	188
	1.0×10^{-4}	90	180
$MgSO_4 \cdot 7H_2O$	1.0×10^{-3}	73	146
	2.0×10^{-3}	81	162
$MnSO_4 \cdot 4H_2O$	3.3×10^{-6}	52	104
	2.0×10^{-5}	62	124
$NiCl_2 \cdot 6H_2O$	3.3×10^{-6}	52	104
	5.0×10^{-4}	21	42
$ZnSO_4 \cdot 7H_2O$	5.0×10^{-4}	18	36
$FeCl_3 \cdot 6H_2O$	3.3×10^{-6}	49	98
	5.0×10^{-4}	50	100
None		64	100
EDTA	1.0×10^{-3}	74	116
α,α′-Dipyridyl	1.0×10^{-3}	74	116
o-Phenanthroline	1.0×10^{-3}	67	105

TABLE IV

Sulfhydryl Character of the Enzyme

Each preincubation was carried out for 5 minutes at 17°. After the third preincubation, the reaction was iniciated with the addition of pyridoxine or TPN. The incubation mixture contained, in a volume of 0.6 ml., 10 μM of pyridoxine, 1.0 μM of TPN, 0.7 mg. of pyridoxine dehydrogenase, 40 μM of sodium carbonate buffer, pH 9.0, and, when indicated, $1 \times 10^{-5} M$ of p-CMB, 3 μM of reduced glutathione. Incubation was carried out for 5 minutes at 37°.

Preincubation			Activity mμM pyridoxal
First	Second	Third	
Enzyme only	p-CMB	TPN	48
Enzyme plus TPN	p-CMB	—	69
Enzyme plus pyridoxine	p-CMB	—	47
Enzyme plus TPN	—	—	79
Enzyme only	—	TPN	95
Enzyme plus p-CMB	Glutathione	TPN	77

glutathione. The dependence of the reaction rate on the glutathione concentration is shown in Fig. 8. The inhibition by p-CMB was completely reversed by glutathione.

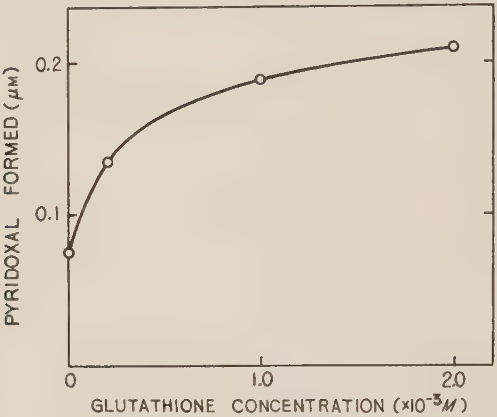


Fig. 8. Effect of reduced glutathione on the enzyme activity.

Each tube contained, in a volume of 1.0 ml., 10 μM of pyridoxine, 1.2 μM of TPN, 0.6 mg. of pyridoxine dehydrogenase, the indicated amount of glutathione and 40 μM of sodium carbonate buffer, pH 9.0, and incubated for 10 minutes at 37°.

TABLE V

Distribution of Pyridoxine Dehydrogenase Activity in Microorganisms

Each microorganism harvested was washed twice with saline and lyophilized.

The incubation mixture contained, in a volume of 1.0 ml., 10 μM of pyridoxine, 1.0 μM of TPN, suspension of each lyophilized cell (10 mg. in dry weight) and 40 μM of sodium bicarbonate buffer, pH 9.0, and was incubated for 30 minutes at 37°.

Microorganism	mμM of pyridoxal formed by 10 mg. of lyophilized cells per hour
Saccharomyces carlsbergensis 4228	46
Streptococcus faecalis R	< 4
Lactobaccilus casei	34
Pseudomonas aeruginosa	26
Baker's yeast	40
Escherichia coli	56

Distribution of the Enzyme Activity—In Table V was presented the distribution of pyridoxine dehydrogenase activity in several microorganisms. Among them, the lowest activity was detected in *Streptococcus faecalis*. This organism was previously shown to lack the pyridoxine phosphate oxidase activity (*2*). These facts support the reported sluggish response of this organism to pyridoxine as a source of vitamin B_6 (*6*).

DISCUSSION

The work presented here shows that baker's yeast contains a TPN-specific pyridoxine dehydrogenase, with a sulfhydryl character, which is activated by some bivalent metal ions. As with other pyridine nucleotide requiring enzymes, the reaction catalyzed by pyridoxine dehydrogenase seems to favor the reduction of pyridoxal at neutral pH.

Bellamy *et al.* (*7*) reported that nicotinic acid and pyridoxine were required for the formation of tyrosine decarboxylase in *Streptococcus faecalis.* This finding suggests pyridine nucleotide participates in the conversion of pyridoxine to pyridoxal phosphate. Recent investigations in our laboratory have revealed that pyridoxine phosphate is oxidized to pyridoxal phosphate by a flavoprotein in rabbit and *Pseudomonas aeruginosa* (*2*). Here it seems that pyridine nucleotide is involved in the oxidation of pyridoxine and flavin nucleotide in the oxidation of phosphorylated pyridoxine in living organisms. There are definitely differences in the metabolism of pyridoxine in various living organisms. For example, the ratio of pyridoxine phosphate oxidase to pyridoxamine phosphate oxidase activity was found to be about 1 in rabbit liver extracts, 2 in *Pseudomonas aeruginosa* extracts, and 0.2 in baker's yeast extracts*. Baker's yeast contained a relatively high-pyridoxine dehydrogenase activity, as compared with the pyridoxine phosphate oxidase activity, suggesting an important role of the former enzyme in the metabolism of B_6 in this organism.

Several B_6 antagonists having a hydroxymethyl group at position-5 of the pyridine ring (*e.g.* 4-deoxypyridoxine, 2-methylpyridoxine, 2-methyl 3-hydroxy 4-methoxymethyl 5-hydroxymethylpyridine) were shown by Hurwitz (*8*) to exert their effects through phosphorylation by pyridoxal kinase, thus leading to an interference with the production of pyridoxal phosphate and/or to the competition of phosphorylated analogues with pyridoxal phosphate for B_6 enzymes. However, the action mechanism of the group of analogues lacking a prerequisit for phosphorylation, such as 5-deoxypyridoxine, 5-deoxypyridoxal, 2-methyl 3-hydroxy 4-hydroxymethylpyridine and 4-nitrosalicylaldehyde, had not been elucidated, although 5-deoxypyridoxal was shown by Snell *et al.* (*9*) to compete with pyridoxal phosphate for alanine aporacemase in *Streptococcus faecalis.* As is clear from the results described above, 5-deoxypyridoxine and 5-deoxypyridoxal are good substrates for pyridoxine dehydrogenase. It is highly probable that the reported growth inhibition of some organisms by these compounds (*10*) is due to their interference with the utilization of B_6 by retarding the conversion of pyridoxine to pyridoxal.

---

* Morino, Y., and Sakamoto, Y., unpublished observations.

SUMMARY

1. Pyridoxine dehydrogenase from baker's yeast was purified about 80-fold over crude extracts.

2. The enzyme required TPN; DPN was not effective.

3. The reaction seems to favor the reduction of pyridoxal.

4. The optimum pH for this enzyme was pH 9.3.

5. The enzymatic activity was stimulated by Co^{++} and Mg^{++}.

6. The enzyme activity was inhibited by p-CMB. The inhibition by p-CMB was prevented by preincubation of the apoenzyme with TPN and reversed by the addition of reduced glutathione.

7. This enzyme seems to be concerned with the oxido-reduction reaction between the hydroxymethyl group and the formyl group at position 4 of pyridine ring.

8. The distribution of pyridoxine dehydrogenase was examined.

9. A possible action mechanism for B_6-antagonists such as 5-deoxypyridoxine and 5-deoxypyridoxal is discussed.

REFERENCES

(*1*) Wada, H., Morisue, T., Nishimura, Y., Morino, Y., Sakamoto, Y., and Ichihara, K., *Proc. Japan. Acad.*, **35**, 299 (1959)

(*2*) Morisue, T., Morino, Y., Sakamoto, Y., and Ichioara, K., *J. Biochem.*, **48**, 18 (1960)

(*3*) Morino, Y., Wada, H., Morisue, T., Sakamoto, Y., and Ichihara, K., *J. Biochem.*, **48**, 28 (1960)

(*4*) Kornberg, A., and Horecker, B. L., in S. P. Colowick and N. O. Kaplan (Editors), "Method in Enzymology," Vol. **I**. Academic Press Inc., New York, p. 323 (1955)

(*5*) Rodwell, V. W., Volcani, B. E., Ikawa, M., and Snell, E. E., *J. Biol. Chem.*, **233**, 1548 (1958)

(*6*) Snell, E. E., and Rannefeld, A. N., *J. Biol. Chem.*, **157**, 457 (1945)

(*7*) Bellamy, W. D., and Gunsalus, I. C., *J. Bacteriol*, **48**, 191 (1944)

(*8*) Hurwitz, J., *J. Biol. Chem.*, **217**, 513 (1955)

(*9*) Olivard, J., and Snell, E. E., *J. Biol. Chem.*, **213**, 203 (1955)

(*10*) Rabinowitz, J. C., and Snell, E. E., *Arch. Biochem. Biophys.* **43**, 408 (1953)

The Journal of Biochemistry, Vol. 48, No. 5, 1960

STUDIES ON TAKA-ACYLASE

II. ACTIVATION BY COBALTOUS ION AND REACTION MECHANISM OF TAKA-ACYLASE

By MASAYORI INOUYE AND IKUNOSHIN KATO

(From the Laboratory of Biochemistry, Faculty of Science, Osaka University, Osaka)

(Received for publication, July 2, 1960)

Metal ions are known to play an important role in various enzymatic reations. Since Smith *et al.* (*1–4*) presented a hypothesis on the role of metal ions in the activity of various metal-activated peptidases, many hypotheses and review articles have been published on this subject (*5–10*). Almost all of them postulated that the metal ion acts as a binding link between the enzyme and the substrate forming an enzyme-metal-substrate complex, which is indispensable for the enzymatic reaction.

Recently Nishi reported on his kinetic studies on the activation of yeast glycylglycine dipeptidase by Co^{++} and elucidated the reaction mechanism (*11*).

Taka-acylase is also activated by Co^{++} as described in the previous paper (*12*).

The present paper describes kinetic investigation on the activation of Taka-acylase by Co^{++} and inhibition of the enzyme by carboxylic acids and *p*-chloromercuribenzoate. From these investigations it is concluded that Co^{++} binds the substrate to the enzyme and with aromatic substrates such as acetyl-L-phenylalanine, the enzyme-metal-substrate complex is stabilized by an interaction of the aromatic side chain of the substrate with a site on the enzyme. This is in contrast to the complex with aliphatic substrate such as chloroacetyl-L-valine. A model of the active area of Taka-acylase is proposed and discussed in the light of the results obtained.

MATERIALS AND METHODS

Enzyme—Taka-acylase was prepared from take-diastase as described in the previous paper (*12*). In the present investigation ,fraction IV in Scheme I of the previous paper (12) was used for kinetic studies. Since this preparation contains excess Co^{++} and sodium acetate the enzyme solution was dialyzed before use as follows: 1 ml. of the enzyme solution was dialyzed two or three times against 1 liter of deionized water for a total period of about 30 hours at 5°.

Measurement of Acylase Activity—Acylase activity was estimated from the amino acids liberated, by the colorimetric ninhydrin method, as described in the previous paper (*12*). The assay system in the present work consisted of 0.3 ml. of the enzyme solution, 0.3 ml. of the enzyme solution, 0.3 ml. of 0.1 *M* veronal-HCl buffer of pH 7.8 or borate buffer pH 8.7, 0.3 ml. of substrate solution and 0.1 ml. of Co^{++} or inhibitor solution. The reaction was started by adding the enzyme solution to the mixture of activator, buffer and substrate which had been preincubated for a short time. As activator $CoCl_2$ was used and as substrate acetyl-L-phenylalanine and chloroacetyl-L-valine were used at a concentration 0.006 to 0.001 *M*. The reaction velocity was expressed in terms of the optical density of ninhydrin positive material at 570 mμ. This was proportional to the concentration of amino acids liberated in the reaction mixture.

---

* A part of this work was presented at the 32nd General Meeting of the Japanese Biochemical Society, Osaka, 1959.

RESULTS AND DISCUSSION

Mechanism of Enzyme Reaction—Using acetyl-L-phenylalanine as substrate (0.006 to 0.001 *M*), the reciprocal of the reaction velocity ($1/v$) was plotted against the reciprocol of the substrate concentration ($1/(S)$) at pH 8.7 in the absence and presence of various concentrations of Co^{++}. As shown in Fig. 1, the results were linear. This may be explained by the following equilibria:

$$\begin{array}{l} E + A \rightleftarrows EA \text{ ---- } K_1 \\ E + S \rightleftarrows ES \text{ ---- } K_2 \\ (A + S \rightleftarrows AS \text{ ---- } K_3) \\ EA + S \rightleftarrows EAS \text{ ---- } K_4 \\ ES + A \rightleftarrows EAS \text{ ---- } K_5 \\ (AS + E \rightleftarrows EAS \text{ ---- } K_6) \end{array} \qquad (1)$$

$$\mathrm{EAS} \xrightarrow{k} \mathrm{E} + \text{product}$$

where *E*, *A* and *S* represent the enzyme, the activator (Co^{++}) and the substrate repectively, and *EA*, *ES* and *AS* represent the various complexes which are formed from these components.

EAS is a complex of the enzyme, activator and substrate. It is postulated that only this complex of the three factors is active for hydrolysis of the substrate. Therefore, *EA*, *ES* and *AS* cannot result in formation of the reoction product without previous interaction with the third factor to form the complex, *EAS* (*S* with *EA*, *A* with *ES* and *E* with *AS*).

In the above formulae, K_1, K_2, K_3, K_4, K_5 and K_6 represent the dissociation constants and each reaction step.

$$K_1 = \frac{(E)\times(A)}{(EA)} \qquad K_4 = \frac{(EA)\times(S)}{(EAS)}$$
$$K_2 = \frac{(E)\times(S)}{(ES)} \qquad K_5 = \frac{(ES)\times(\mathrm{A})}{(EAS)} \qquad (2)$$
$$K_3 = \frac{(A)\times(S)}{(AS)} \qquad K_6 = \frac{(AS)\times(E)}{(EAS)}$$
$$K_1K_4 = K_2K_5 = K_3K_6$$

Now, assuming that in a stationary state, the reaction rate is proportional to the concentration of the complex, *EAS*, and designating the reaction velocity by v and the velocity constant of the reaction ($EAS \longrightarrow E + \text{product}$)

by k, the following formula can be derived from the above equations:

$$v=k\ (EAS)=\frac{k\ (e)\ (a)\ (s)}{K_1K_4+K_4\ (a)+K_5\ (s)+(a)\ (s)} \tag{3}$$

where (e), (a) and (s) represent the total concentrations of enzyme, activator and substrate, respectively. In this formula, the following assumption has been introduced. Acyl amino acids scarcely form a complex with metal ions such as Co^{++}, in contrast to the amino acids with free amino groups. Therefore the reactions designated by K_3 and K_6 may be neglected in the reaction system. In other words (AS) may be negligibly small and furthermore the concentration of enzyme is also virtually negligible in comparison with that of the activator and the substrate, thus

$$(e)=(E)+(ES)+(EA)+(EAS)$$
$$(a)=(A)+(EA)+(AS)+(EAS)=(A)$$
$$(s)=(S)+(ES)+(AS)+(EAS)=(S)$$

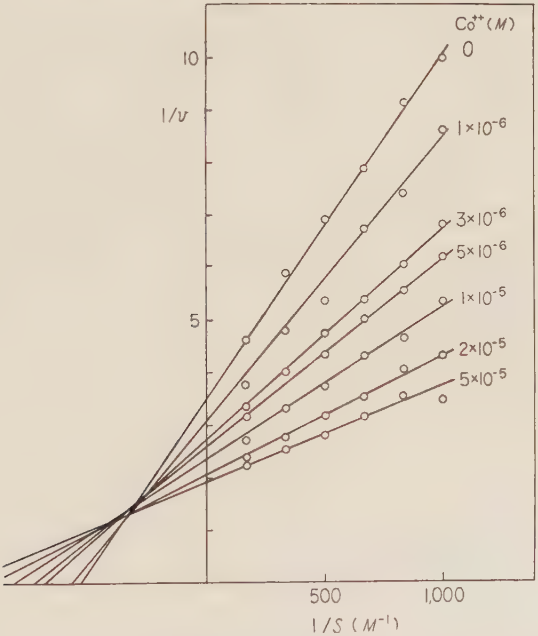


FIG. 1. Lineweaver-Burk plot for acetyl-L-phenylalanine with Taka-acylase at pH 8.7 in the absence and presence of various concentration of Co^{++}.

Equation (3) can be rewritted as follows:

$$\frac{1}{v}=\frac{1}{k\ (e)}\left\{\left(\frac{K_1K_4}{(A)}+K_4\right)\frac{1}{(S)}+\frac{1}{(A)}+1\right\} \tag{4}$$

Therefore when $1/v$ is plotted against $1/(S)$, a straight line will be obtained in accordance with Equation (4). The results obtained in Fig. 1, may be explained by Equation (4). But in this case a straight line was obtained even if Co^{++} was not added to the reaction mixture. This may be explained by assuming that there were traces of Co^{++} which could not be eliminated by dialysis under the conditions used or which were present as impurities in the buffer. Thus, assuming that the concentration of this trace of Co^{++} is (A_0), its value can be calculated from the values of the slopes or intercepts on the vertical axis of the straight lines in Fig. 1, in accordance with Equation (4). By the least square method, using the values of the intercepts, (A_0) was calculated to be $3.5 \times 10^{-6} M$. With this value, $1/V_{max.}$* (itercept on the vertical axis of the straight lines in Fig. 1) and the value of the slope of the straight lines in Fig. 1 were replotted against $1/(A)$ (herein $(A)=(A_0)+(A_{added})$). The assumption introduced in the hypothesis is correct since straight lines were obtained as shown in Fig. 2 and 3.

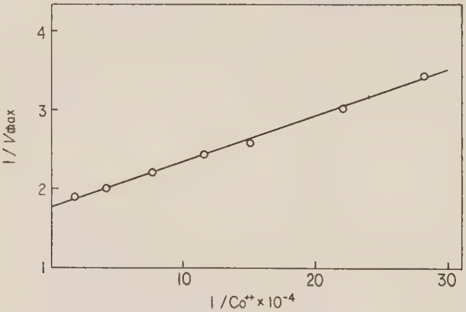


Fig. 2. Relationship between $1/(A)$ and $1/V_{max.}$ in the straight lines in Fig. 1.

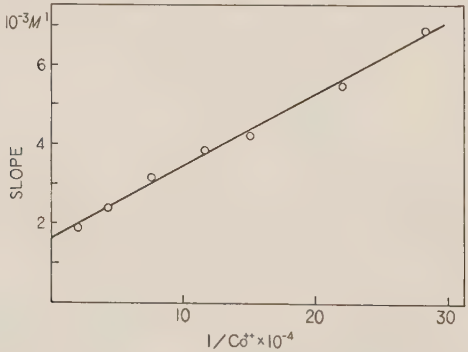


Fig. 3. Relationship between $1/(A)$ and the slope in the straight lines in Fig. 1.

Now, according to Equation (4), the slope of the the straight line in Fig. 2 is equal to K_5:

$$K_5 = 3.2 \times 10^{-6} \text{ moles/liter},$$

and the intercept on the vertical axis gives a value for $1/V_{max.}$ -Co-sat., where $V_{\max.}$ -Co-sat. is the maximal reaction velocity when the concentration of Co^{++} becomes infinitely high:

---

* V_{max}: the maximal reaction velocity.

$$V_{max.}\text{-Co-sat.}=k(e)=5.6\times10^{-1}\ \text{(arbitrary units)}$$

On the other hand, the itercept of the straignt line in Fig. 3 and the slope of the line equal to $K_4/V_{\max.}$ -Co-sat. and $K_1K_4/V_{\max.}$ -Co-sat. respectively:

$$K_4=8.4\times10^{-4}\ \text{moles/liter}$$

and $K_1K_4=9.96\times10^{-9}$ (moles/liter)^2 therefore K_2 and K_1 can be calculated as:

$$K_2=\frac{K_1K_4}{K_5}=3.2\times10^{-3}\ \text{moles/liter}$$

and

$$K_1=\frac{K_1K_4}{K_4}=1.2\times10^{-5}\ \text{moles/liter}$$

For K_1, the dissociation constant of the reaction $(E+A=EA)$, Nishi (*11*) obtained a value of 4.8×10^{-4} moles/liter for glycylglycine dipeptidase, an enzyme which is also activated by Co^{++}.

A similar kinetic analysis was undertaken, using chloroacetyl-L-valine instead of acetyl-L-phenylalanine as substrate. But in this case it is uncertain whether the same enzyme and furthermore the same active site of the enzyme participates in the hydrolysis of both acetyl-L-phenylalanine and chloroacetyl-L-valine. This problem was discussed in the previous paper and it was suggested from the results of zone electrophoresis and heat denaturation that both activities were due to the same enzyme. In the present paper the problem was examined kinetically as follows: As already reported by Dixon (*13*), when an enzyme acts on two substrates A and B, through the same active site, the reaction velocity in a mixture of A and B is given as:

$$V_m=\frac{\dfrac{Va.a}{Ka}+\dfrac{Vb.b}{Kb}}{1+\dfrac{a}{Ka}+\dfrac{b}{Kb}} \tag{5}$$

where a and b are the concentrations of A and B, and Va and Vb and Ka Kb are the maximal reaction velocity and dissociation constant of the following equilibrium:

$$E+A\ (\text{or } B)\rightleftarrows EA\ (\text{or } EB)$$

When B is present at n times the concentration of A $(b=na)$, the above equation (5) can be rewritten as follows:

$$\frac{1}{v_m}=\frac{KaKb}{VaKb+nVbKa}\cdot\frac{1}{a}+\frac{Kb+nKa}{VaKb+nVbKa} \tag{6}$$

According to this equation, $1/v_m$ has a linear relationship to $1/a$ and the intercept on the vertical axis and the slops must be given by $(Kb+nKa)/(VaKb+nVbKa)$ and $KaKb/(VaKb+nVbKa)$ respectively, if the enzyme acts on A and B at the same time.

This was applied to Taka-acylase and the results are shown in Fig. 4, where A and B represent acetyl-L-phenylalanine and chloroacetyl-L-valine respectively and Ka, Kb, Va and Vb were calculated as 1.7×10^{-3} moles/liter, 1.9×10^{-2} moles/liter, 6.5×10^{-1} and 4.8×10^{-1} (arbitrary units) respectively from the results in Fig. 4. The experiments were carried out for $n=1$ and $n=5$.

As shown in Fig. 4, there is quite good agreement between the calculated and observed values for both $n=1$ and $n=5$. From these results it is evident that the same active site of Taka-acylase reacts with the two substrates.

Therefore, just as for acetyl-L-phenylalanine, $1/v$ was plotted against $1/(S)$ for chloroacetyl-L-valine at various concentration of Co^{++}. As shown in Fig. 5 straight lines were obtained but all the lines cut the vertical axis at the same point, in contrast to the case with acetyl-L-phenlalanine. This may be accounted for as follows.

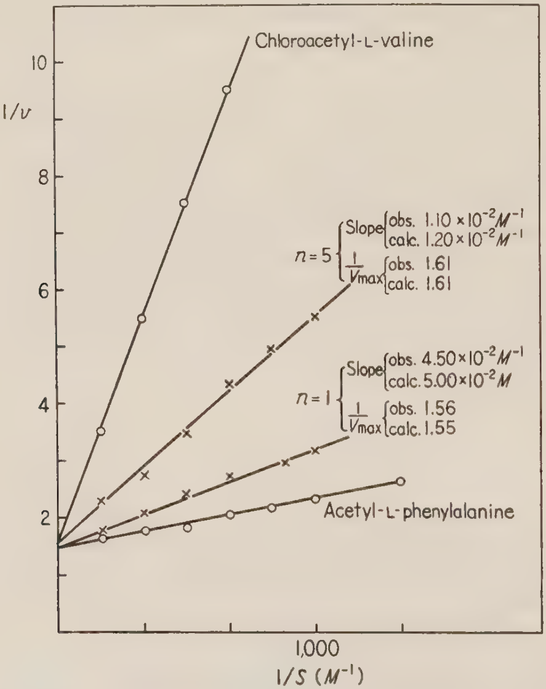


FIG. 4. Lineweaver-Burk plot for acetyl-L-phenylanine and chloroacetyl-L-valine and reciprocal of reaction velocity as a function of that of the concentration of acetyl-L-phenylanine in the presence of both acetyl-L-phenylalanine and chloroacetyl-L-valine. The experiments were carried out at pH 7.8 in the presence of $5\times10^{-4}\,M$ Co^{++}.
The slopes and $1/V_{max}$. were calculated according to Equation (7).

Assuming that chloroacetyl-L-valine itself cannot bind the enzyme in the absence of the activator, Co^{++}, the equilibrium deisgnated by K_2 can be eliminated form Equilibria (1). Therefore the reciprocal of the reaction velocity is given by:

$$\frac{1}{v}=\frac{1}{k(e)}\left\{\left(\frac{K_1K_4}{(A)}+K_4\right)\frac{1}{S}+1\right\} \qquad (7)$$

Thus when $1/v$ is plotted against $1/(S)$ at various concentrations of activator, straight lines will be obtained and all lines will cut the vertical axis at the same point ($1/k(e)$) according to Equation (7).

The experiments were carried out under the same condition as for acetyl-L-phenylatanine. Using acetyl-L-phenylanine, (A_0) was calculated as mentioned above to be $3.2\times10^{-6}\,M$. The enzyme was used at the same concentration as for acetyl-L-phenylalanine. On the basis of the value, (A_0), the slopes of the straight lines in Fig. 5 were replotted against 1/(A) (Fig. 6). As shown in Fig. 6, straight lines were obtained. According to Equation (7), K_1K_4 and K_4 were calculated from the slope and intercept on the vertical axis as:

$$K_1K_4=1.28\times10^{-6}\ (\text{moles/liter})^2$$

and

$$K_4=4.1\times10^{-2}\ \text{moles/liter}$$

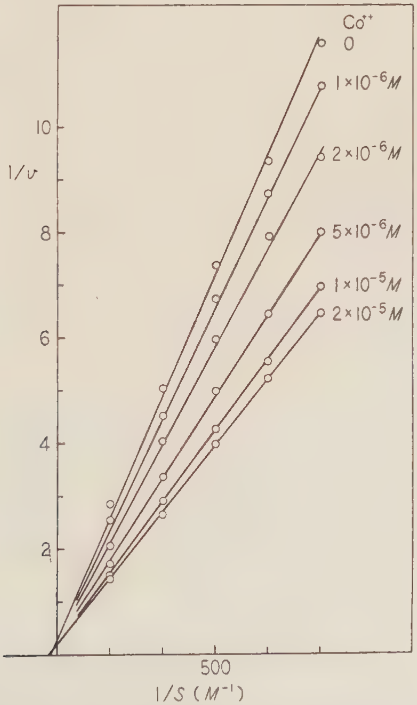


FIG. 5. Lineweaver-Burk plot for chloroacetyl-L-valine with Taka-acylase at various concetrations of Co^{++} at pH 8.7.

therefore $K_1 = K_1K_4/K_4 = 4.1 \times 10^{-5}$ moles/liter. K_1, which is the dissociation constant of the enzyme-activator complex, was found to be in the same order as that obtained with acetyl-L-phenylalanine as substrate. Furthermore K_4, which is the dissociation constant of EA and S, is 8.4×10^{-4} moles/liter for acetyl-L-phenylalanine and 4.1×10^{-2} moles/liter for chloroacetyl-L-valine. These facts support the above assumption.

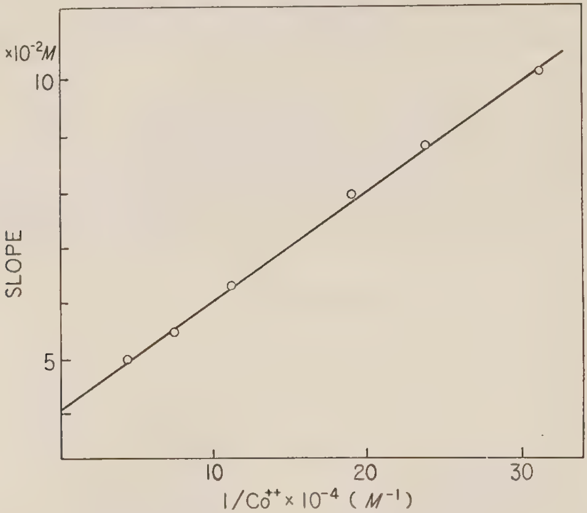


FIG. 6. Relationship between $1/(A)$ and the slopes of the straight lines in Fig. 5.

The assumption that the ES complex is not formed with chloroacetyl-L-valine, may be explained as follows. Aromatic substrates such as acetyl-L-phenylalanine can from the ES complex by the interaction of their benzene rings with a site in the active area of Taka-acylase, having affinity for the benzene ring of the substrate. But aliphatic substrates such as chloroacetyl-L-valine cannot form such a complex because they have no benzene ring.

Inhibition by Carboxylic Acids—Hata *et al.* (*14*) investigated the inhibition of acylase from the culture medium of *Aspergillus oryzae* by various carboxylic acids and measured the pK_i values of these acids. They showed that β-phenylpropionic acid inhibits acylase action non-competitively and suggested that these inhibitory acids were bound to the site on the enzyme to which the acyl group of the substrate is attracted when an active complex is formed.

But, as shown in Fig. 7, acetate inhibited acylase action "anti-competitively" rather than non-competitively. Thus, in the presence of sodium acetate, when $1/v$ was plotted against $1/(S)$ or $1/(A)$ straight lines with the same slopes as their controls were obtained. This can be explained by assuming that acetate inhibits acylase action by binding only the triple complex, *EAS*, forming an inactive complex, *EASI*. Therefore, besides Equilibria (1) there is an additional one:

$$\mathrm{EAS} + \mathrm{I} \overset{K_i}{\rightleftharpoons} \mathrm{EASI}$$

where I and K_i represent the inhibitor concentration and the dissociation constant of the equlibrium respectively. Thus, when acetyl-L-phenylalanine is used as substrate, the reciprocal of the reaction velocity is given by.

$$\frac{1}{v}=\frac{1}{k(e)}\left\{\left(\frac{K_1K_4}{(A)}+K_4\right)\frac{1}{(S)}+1+\frac{K_5}{(A)}+\frac{I}{K_i}\right\}$$

or

$$\frac{1}{v}=\frac{1}{k(e)}\left\{\left(\frac{K_1K_4}{(S)}+K_5\right)\frac{1}{(A)}+1+\frac{K_4}{(S)}+\frac{I}{K_i}\right\}$$

According to these equations, the difference in the intercept on the

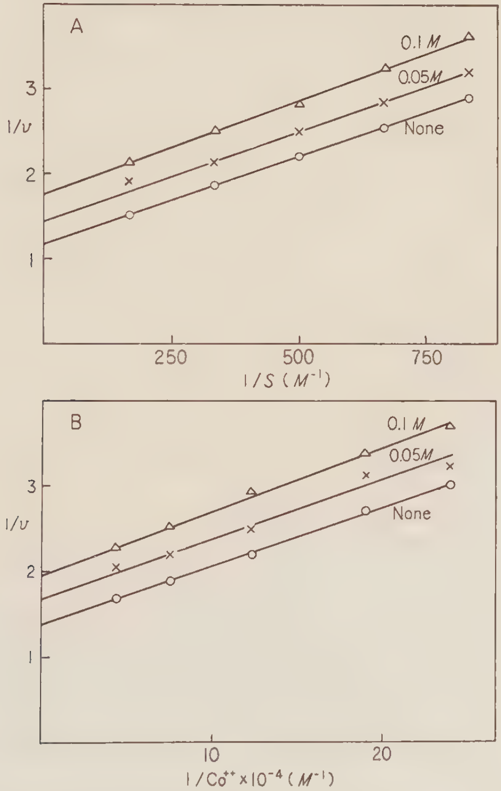


FIG. 7. Inhibition of acylase activity by sodium acetate at pH 8.7. Acetyl-L-phenylalanine was used as substrate. Experiments were performed under following conditions:

A: at various concentrations of the substrate in the presence of $2\times10^{-5}\,M$ Co^{++}.

B: at various concetrations of Co^{++} in the presence of $0.003\,M$ substrate.

vertical axis is equal to $1/K_i \cdot k(e)$. From the results in Fig. 7, K_i was calculated respectively as 0.15, 0.18, 0.14 and 0.13 moles/liter for 0.1 *M*, and 0.05 *M* sodium acetate in Fig. 7, A, and for 0.1 *M* and 0.05 *M* acetate in Fig. 7, B. Therefore the average value for pK_i is 0.82. When propionic acid was used, similar results were obtained and the pK_i of this acid was 1.2.

Results on the inhibition by β-phenylpropionic acid are shown in Fig. 8. The pK_i for this acid was calclated, assuming non-competitive inhibition as 2.27 for A and 2.32 for B in Fig. 8 respectively. A rather large difference in the pK_i value between β-phenylpropionate and acetate or propionate was observed. This agrees with the results obtained by Hata *et al.* (*14*). However, this difference cannot be explained by a difference in the pK_a (acid dissociation constant) of these acids, because the pK_a of β-phenylpropionate, acetate and propinate are nearly the same at 25°, namely

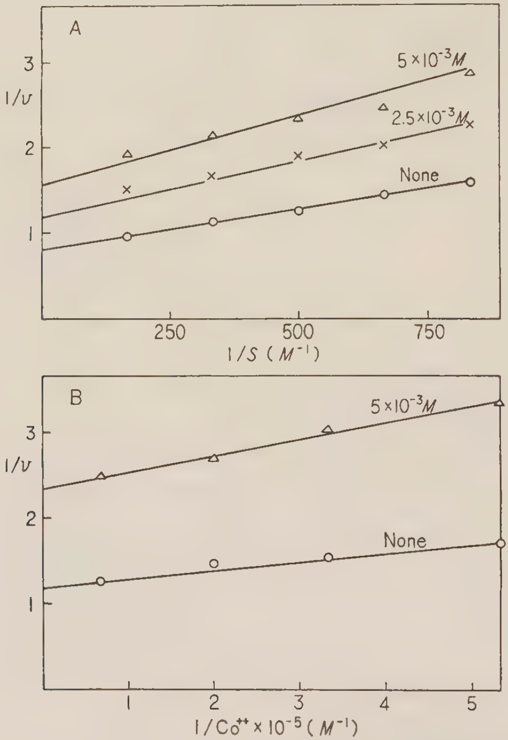


FIG. 8. Inhibition of acylase activity by β-phenylpropinic acid at pH 8.7. Acetpl-L-phenylalanine was used as substrate. Experiments were performed under the following conditions :

A : at various concentrations of substrate in the presence of 2×10^{-5} *M* $Co^{+\prime}$.

B : at various concentration of Co^{++} in the presence of 0.003 *M* substrate.

4.64, 4.85 and 4.76 respectively. It may be accounted for by the assumption introduced previously, that inhibitory aromatic acids may interact with a site on the enzyme which has an affinity for the benzene ring in contrast to aliphatic acids. Thus, they may inhibit the enzyme at a lower concentration than aliphatic acids.

Inhibition by p-Chloromercuribenzoate (*PCMB*)—As mentioned in the previous paper (*12*) acylase activity is inhibited by PCMB. Kinetic analysis of the inhibition by PCMB showed that it acted as a competitive inhibitor of 1/(A), as shown in Fig. 9. Therefore PCMB must combine with the sulfhydryl group, which is one of the sites on the enzyme responsible for binding Co^{++}.

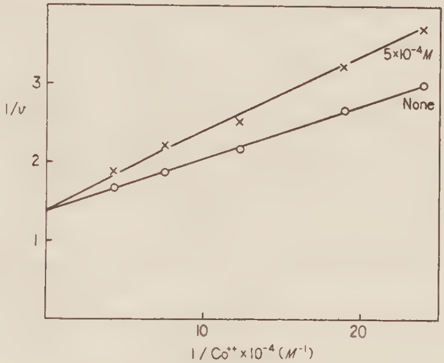


FIG. 9. Inhibition of acylase activity by *p*-chloromercuribenzoate at pH 8.7. 0.03 *M* acetyl-L-phenylalanine was used as substrate.

Inhibition by Other Substance—Chloroacetyl-D-valine, carbobenzoxyglycyl-L-leucine and benzoyl-L-phenylalanine scarcely inhibited acylase activity at a concentration of 0.005 *M*.

The Active Area of Taka-Acylase—From the results obtained, the active area of Taka-acylase may be as shown in Fig. 10: Co^{++} mediates the binding of the substrate to the enzyme and one of the sites which is responsible for the binding between Co^{++} and the enzyme seems to be a sulfhdyryl group. On the substrate, the metal ion combines with acylated amino nitrogen and forms an electrostatic bond with the carboxyl group. Furthermore with acetyl-L-phenylalanine, the phenyl group of the substrate interacts with a site on the enzyme designated by X in Fig. 10 to stabilize the complex. Mor experimental data are necessary to elucidate the reaction mechanism, but by comparison with Rabin's theory on the reaction mechanism of prolidase (*9*), the reaction mechanism of acylase may be considered as shown in Fig. 10. Besides the interactions mentioned above, additional interactions may be involved at disignated by AH and B^-. The former transfers a proton to the substrate nitrogen and the latter assists the nucleophilic attack by a water molecule. Thus sodium acetate or propionate

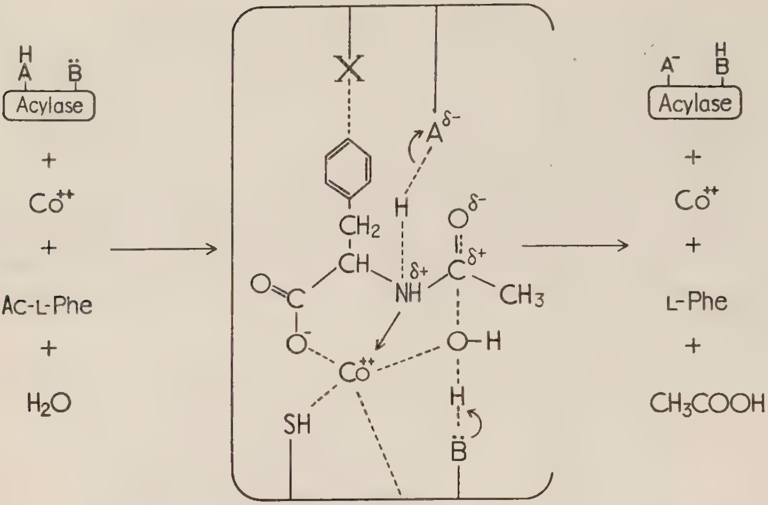


FIG. 10. The active area of Taka-acylase and a possible mechanism for the acylase reaction.
Ac-L-Phe. : Acetyl-L-phenylalanine.
L-Phe. : L-phenylalanine.

may act here intead of a water molecule to inhibit the activity anti-competitively.

SUMMARY

The activation of Taka-acylase by Co^{++} was invesigated kinetically. A scheme for the acylase reaction is proposed and it is concluded that Co^{++} mediates in the binding of the substrate to the enzyme and that with aromatic substrates such as acetyl-L-phenylalanine, the enzyme-metal-substrate complex is stabilized by the interaction of the benzene ring of the substrate with a site of the enzyme. This is in contrast to the reaction with aliphatic substrates such as chloroacetyl-L-valine.

Inhibition by carboxylic acids and *p*-choloromercuribenzoate was also investigated kinetically.

A model of the active area of Taka-acylase is proposed and discussed.

The authors wish to express their deep gratitude to Dr. S. Akabori, Prof. of Institute for Protein Research, for his kind guidance and Dr. Y. Ogura, Prof. of the University of Tokyo, for his valuable discussion throughout this investigation and also to Sankyo Co., Ltd. for kindly supplying "Taka-diastase Sankyo".

REFERENCES

(*1*) Smito, E. L., *J. Biol. Chem.*, **173**, 571 (1948)

(*2*) Smith, E. L. and Lumry, R., *Cold Spring Harbor Symposia Quant. Biol.*, **14**, 168 (1950)

(*3*) Smith, E. L., "Enzymes and Enzyme Systems," Ed. by Edsall, J. T., Har-

vard Univ. Press, p. 47 (1951)
(*4*) Smith, E. L. and Speckman, D. H., *J. Biol. Chem.* **212**, 271 (1955)
(*5*) Najjar, V. A., "*Phosphorous Metabolism*," The Johns Hopkins Press, Baltimore, **1**, 500 (1951)
(*6*) Lehninger, A. L., *Physiol. Rev.*, **30**, 393 (1950)
(*7*) Williams, R. J. P., *Biol. Rev.*, **28**, 381 (1953)
(*8*) Vallee, B. L., *Adv. Protein Chem.*, **10**, 317 (1955)
(*9*) Rabin, B. R., *Biochem. Soc. Symposia.*, No. 15, p. 31 (1958)
(*10*) Dixon, M. and Walb, E. L., "Enzymes" Longman, Green and Co, p. 355 and 448 (1958)
(*11*) Nishi, A., *J. Biochem.*, **45**, 991 (1958)
(*12*) Inouye, M. and Akabori, S., *J. Biochem.*, **48**, 603 (1960)
(*13*) Dioxon, M. and Walb, E. L., "Enzymes," Longman, Green and Co, p. 91 (1958)
(*14*) Hata, T., Doi, E. and Asao, T., *Symposium on Enzyme Chem.*, **14**, in press.

The Journal of Biochemistry, Vol. 48, No. 5, 1960

ENZYMATIC STUDIES ON THE PHOSPHORUS METABOLISM IN GERMINATING SPORES OF ASPERGILLUS NIGER*

By ARASUKE NISHI

(*From the Institute of Food Microbiology, Chiba University,*
Okubo, Narashino, Chiba-ken)

(Received for publication, July 12, 1960)

Previous papers (*1*, *2*) have presented evidence that the phosphorus metabolism of conidiospores of *Aspergillus niger* plays an important role in the initiation of germination. Thus the polyphosphate and phospholipid contained in dormant spores are shown to be the source of phosphorus, which in turn is utilized for the formation of nucleotides, sugar phosphates, and other phosphorus compounds.

In connection with these findings, experiments were conducted to extend the enzymatic background of the phosphorus metabolism in germinating spores of the fungus. Of various enzymes studied, polyphosphate-kinase was found to be especially interesting because of its increse in activity in the initial phase of germination, since it was already found that polyphosphate phosphorus contained in dormant spores was transferred to various nucleotides (*2*).

MATERIALS AND METHODS

Preparation of Cell Free Extract—Spores of *Aspergillus niger* strain 1617 were collected by the dry preparation method of Yanagita and Yamagishi (*3*), and grown in germination medium as described previously. Spores harvested at various time intervals were suspended in water and disrupted by sonic vibration in the presence of glass beads for 15 minutes with a 10 KC magnetostriction oscillator in the cold. The extract was centrifuged at about 600×*g* for 5 minutes to remove cellular debris. In all experiment freshly prepared cell-free extracts were used as enzyme preparations. The protein nitrogen content of a precipitate (with hot 5 per cent trichloroacetic acid) of the enzyme preparation was estimated, after Kjeldahl digestion, by the method of Akamatsu and Katsumata (*4*).

Measurement of Hydrolytic Enzyme Activities—Pyrophosphatase, phosphomonoesterase, apyrase, and polyphosphatase activities were determined by estimation of the orthophosphate liberated from appropriate substrates, employing the method of Fiske and SubbaRow (*5*). Polyphosphatase activity was also determined viscosimetrically by means of Ostwald's viscosimeter. All the enzyme activities were expressed, unless otherwise specified, in terms of the amount of product formed per 10 minutes per μg. protein

---

* This work was supported in part by grants from the Ministry of Education, and from the Waksman Foundation of Japan donated to Dr. T. Yanagita.

nitrogen of enzyme preparation. These enzymatic reactions were carried out at 30°.

Measurement of Polyphosphate-kinase Activity—Enzymatic transfer of polyphosphate phosphorus to adenosine diphosphate (ADP), to yield adenosine triphosphate (ATP) was determined, using P^{32}-labeled polyphosphate, by measuring the radioactivity of the ATP isolated from the reaction system. The radioactive polyphosphate used as the substrate in this reaction was prepared by the method described by Kornberg (*6*). After the incubation of 1 ml. reaction mixture at 30°, 0.2 ml. of egg albumin (50 mg./ml.) and 1 ml. of cold perchloric acid (10 per cent) solution were added to remove the remaining polyphosphate and proteinacious substances. After neutralization with KOH, the resulting supernatant was lyophilized and the residue dissolved in 0.1 ml. of water. An aliquot (0.05 ml. for quantitative test) of the solution was paper-chromatographed with a solvent system of *iso*-propanol-trichloroacetic acid—NH_4OH (*7*). Spots located by autoradiography and ultraviolet absorption were cut out from the paper and eluted with 0.01 N HCl for measurement of their radioactivities.

RESULTS

Pyrophosphatase—Spore extract was found to exhibit a considerable activity of pyrophosphatase throughout the course of germination. The activity-pH curve for the enzymatic hydrolysis of pyrophosphate showed two maxima, at pH 6.0 and 7.5, suggesting the presence of at least two types of enzymes (Fig. 1). Both enzymes were activated to a great extent

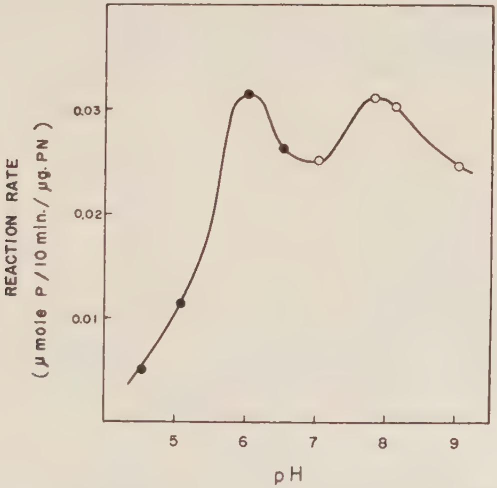


FIG. 1. pH-activity curve of enzymatic hydrolysis of pyrophosphate by spore extract of *Aspergillus niger*. Reaction mixture (5 ml.) contained $3 \times 10^{-2} M$ acetate or Tris buffer, $10^{-3} M$ sodium pyrophosphate, $10^{-3} M$ $MgCl_2$ and spore extract (2.7 μg. protein nitrogen per ml. reaction mixture). At various time intervals, 1 ml. aliquots were removed and orthophosphate content determined. Reaction rate at various pH's were determined from time-course curves of reaction. P and PN represent, respectively, orthophosphate and protein nitrogen. —●—, in acetate buffer; —○—, in Tris buffer.

by Mg ion but not by Mn ion. As shown in Fig. 2, the activities of the

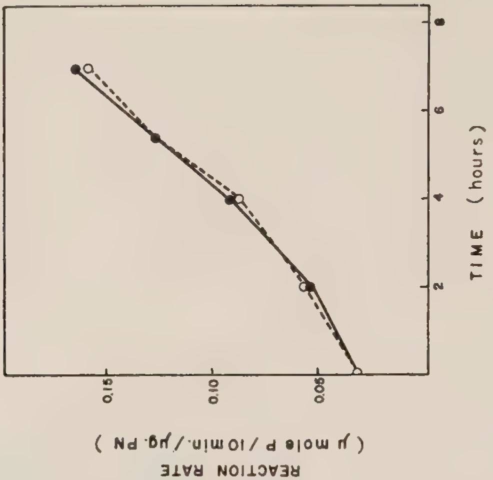


Fig. 2. Change in pyrophosphatase activity of spore extract of *Aspergillus niger* during course of germination. Experiments were performed as described in Fig. 1 in presence of 10^{-3} *M* sodium pyrophosphate and 10^{-3} *M* $MgCl_2$. —●—, at pH 6.0 in acetate buffer; —○—, at pH 7.8 in Tris buffer.

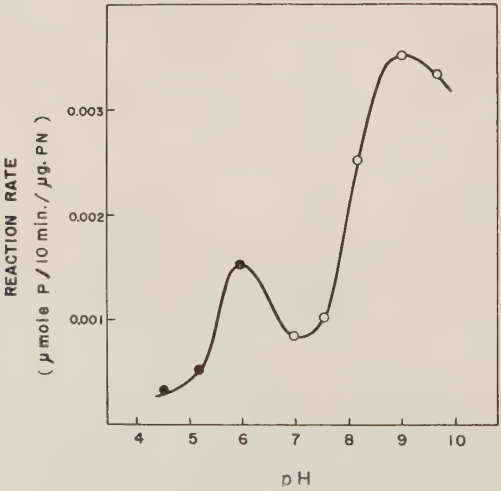


Fig. 3. pH-activivy curve of enzymatic hydrolysis of β-glycerophosphate by spore extract of *Aspergillus niger*. Reaction mixture (5 ml.) contained 3×10^{-2} *M* acetate or Tris buffer, 2×10^{-2} *M* sodium β-glycerophosphate, 5×10^{-3} *M* $MgCl_2$ and spore extract (10.8 μg. protein nitrogen per ml. reaction mixture). Experiments were performed as described in Fig. 1. —●—, in acetate buffer; —○—, in Tris buffer.

two types of pyrophosphatases were found to increase markedly during the course of germination. Thus, at the 7th hour of germination, the enzyme activities of germinating spores were about 6 times those of dormant spores. Even at this stage of germination, the shape of the activity-pH curve of pyrophosphatase was found to be essentially unchanged.

Phosphomonoesterase—The phosphomonoesterase activity of the spore extract was measured, using β-glycerophosphate as the substrate in the presence of $MgCl_2$. Judging from the shape of the activity-pH curve (Fig. 3), two types of enzymes hydrolyzing phosphomonoester are also present. The activity of an enzyme showing a maximum at pH 6.0 increased slightly during the course of germination, while that of alkaline esterase, having maximal activity at pH 9.0, showed a gradual decrease (Fig. 4).

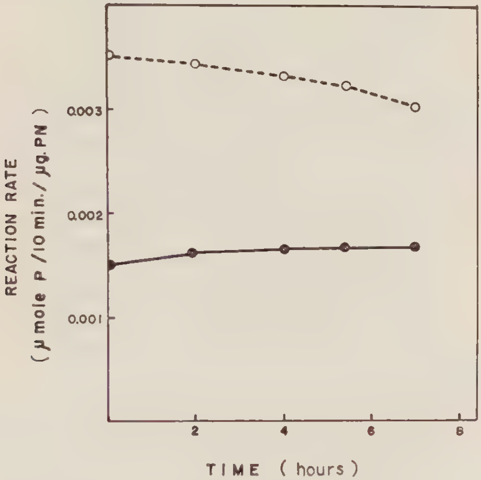


FIG. 4. Change in phosphomonoesterase activity of spore extract of *Aspergillus niger* during course of germination. Experiments were performed as described in Fig. 1 in presence of $2\times10^{-2}\,M$ β-glycerophosphate and $5\times10^{-3}\,M$ $MgCl_2$. —●—, at pH 6.0 in acetate buffer; —○—, at pH 9.0 in Tris buffer.

Apyrase—The activity of an enzyme producing orthophosphate from ATP was studied in the presence of $CaCl_2$ or $MgCl_2$ as an activator. Little or no enzymatic activity was observed in spore extracts obtained at various stages of germination under the present experimental conditions, although in preliminary studies using a rather large amount of spore extract, the maximal activity seemed to be exhibited at around pH 6.0.

Polyphosphatase—In the viscosimetric analysis of the polyphosphate depolymerizing activity of the spore extract, the time required for obtaining half viscosity ($t_{\frac{1}{2}}$) of the substrate solution was found to be inversely proportional to the enzyme concentration (Table I). The activity of this enzyme was, therefore, expressed arbitrarily in terms of $1/t_{\frac{1}{2}}$ per μg. of

TABLE I

Reciprocal Relationship between Polyphosphatase Concentration and Time Required for Obtaining Half-viscosity ($t_{\frac{1}{2}}$) of Reaction Mixture

Enzyme concentration		$t_{\frac{1}{2}}$ minutes
µg. protein-N in ml. reaction mixture	relative value	
32.0	1.0	5.1
16.0	0.5	10.2
9.6	0.3	17.0
4.8	0.15	34.1

Experiments were performed in presence of 10^{-2} *M* sodium polyphosphate, 10^{-3} *M* $MgCl_2$ and spore extract in 2×10^{-2} *M* acetate buffer (pH 6.0). Values of $t_{\frac{1}{2}}$ were determined from time-course curve of viscosity change.

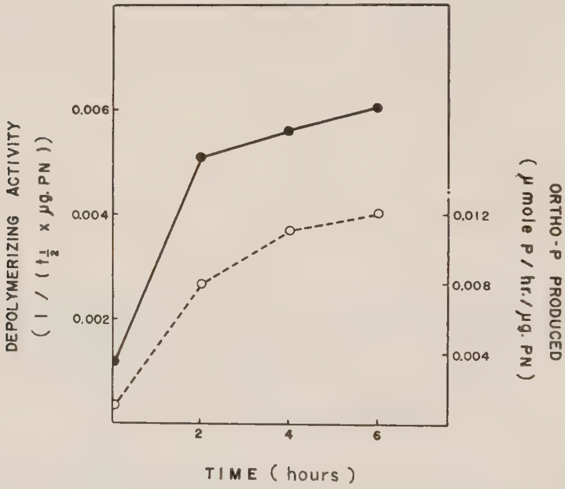


FIG. 5. Change in polyphosphate hydrolyzing activity of spore extract of *Aspergillus niger* during course of germination. Reaction mixture (5 ml.) contained 2×10^{-2} *M* acetate buffer (pH 6.0), 8×10^{-3} *M* sodium polyphosphate, 10^{-2} *M* $MgCl_2$ and spore extract. In viscosimetric analysis, values of $t_{\frac{1}{2}}$ were determined from time-course curve of viscosity change. Enzyme concentration used at each estimation was regulated to give common $t_{\frac{1}{2}}$ value around 15 minutes. In estimation of orthophosphate production, 1 ml. aliquot was removed from each reaction mixture after 1 hour incubation and orthophosphate content determined. —●—, viscosity change; —○—, orthophosphate production.

protein nitrogen of spore extract. As shown in Fig. 5, the polyphosphate depolymerizing activity increased markedly, especially in the early phase of germination. During this short period, the activity reached a level about 5 times that of dormant spores; a course of less marked activity increase followed. In parallel with the viscosimetric analysis, the production of orthophosphate from the substrate was also measured. In this experiment the reaction was terminated after 1 hour, during which period the viscosity of the reaction system fell to almost a minimum. As clearly demonstrated in Fig. 5, the time course of the activity of orthophosphate liberation was found to run almost parallel with that of viscosity change. It is, however,

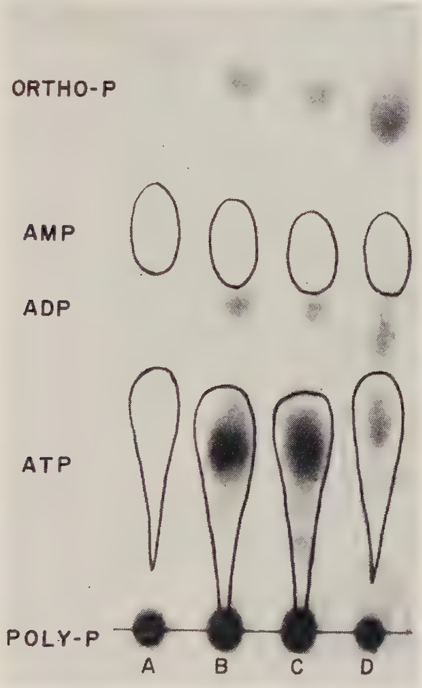


Fig. 6. Demonstration of presence of polyphosphate-kinase activity in spore extract of *Aspergillus niger* by autoradiography. P^{32}-polyphosphate (2.2×10^{-3} M, 1,025 c.p.m. per μmole P) was incubated with 10^{-3} M adenylnucleotides, 2×10^{-3} M $MgCl_2$ and spore extract (36 μg. protein nitrogen per ml. reaction system) at pH 7.0 for 30 minutes. Reaction was stopped by addition of perchloric acid and resultant supernatant was neutralized with KOH and subjected to chromatography. A, without addition of adenylnucleotide; B, with ATP; C, with ADP; D, with AMP. Outlined spots represent carrier ATP and AMP detected by ultraviolet absorption.

important that the percentage of polyphosphate phosphorus converted to orthophosphate was found not to increase above 0.1 per cent even at a later stage of germination. This shows that the spore extract contain an enzyme which mainly catalizes a reaction splitting macro-molecular weight polyphosphate, but not into orthophosphate. To confirm this, a qualitative analysis of the reaction product was carried out by means of paper chromatography, using P^{32}-labeled polyphosphate as the substrate. After the viscosity of the reaction mixture decreased to one-tenth of the initial value, the reaction was stopped by the addition of cold perchloric acid, and the mixture was treated as described previously to remove macromolecular polyphosphate and protein. The supernatant solution thus obtained was subjected to paper chromatography. As can be seen in Fig. 6A, almost all the radioactivity remained at the origin. although a faint spot of orthophosphate appeared at the top. No low molecular weight polyphosphates, such as pyro- or tri-phosphate, were detected. From this observation, the reaction products of the depolymerase may be considered to have a degree of polymerization of not less than Graham's salt (*7*).

Polyphosphate-kinase—Enzymatic transfer of phosphorus from polyphosphate to ADP, yielding ATP, was studied using P^{32}-labeled polyphosphate. A preliminary qualitative observation was carried out, employing cell-free extracts of dormant spores. An autoradiogram of the reaction products formed under various experimental conditions is shown in Fig. 6. As may be seen in the figure, the extract of dormant spores showed a definite activity of transferring phosphorus from polyphosphate to adenylnucleotides, yielding ATP. Among the nucleotides tested, ADP appeared to be the most effective as the phosphorus acceptor from polyphosphate. Adenosine-5′-monophosphate (AMP) and ATP showed less reactivity in this system.

TABLE II

Enzymatic Synthesis of P^{32}-ATP from P^{32}-Polyphosphate and ADP by Cell-free Extract of Germinating Spores of Aspergillus niger

Spore extract	Phosphorus transferred to ADP after 60 min. incubation		
	per tube		per μg. protein nitrogen
	c.p.m.	μmole	μmole
0 time culture	160	0.7	0.013
3 hour culture	280	1.2	0.030

Reaction mixture (1 ml.) contained 3.3×10^{-2} *M* Tris buffer (pH 7.0), 2.2×10^{-2} *M* sodium P^{32}-polyphosphate (232 c.p.m. per μmole P), 2×10^{-3} *M* ADP, 2×10^{-3} *M* $MgCl_2$ and spore extract. Enzyme concentrations in terms of protein nitrogen were 54 and 44 μg. per ml. reaction mixture for 0 time and 3 hour culture, respectively.

Under the present experimental conditions, it cannot be concluded whether or not the latter two nucleotides are true substrates in this enzyme system, since many other enzymes including phosphokinases may be contained in the spore extract. These observations, however, clearly demonstrate that ADP serves as a phosphorus acceptor from polyphosphate in this reaction system. Therefore, estimation of the enzyme activity using ADP as an acceptor was performed, employing cell-free extracts of dormant spores and of 3rd hour germinating spores. Reaction mixture containing P^{32}-labeled polyphosphate were treated as described previously, and the radioactivity of the ATP formed was measured. The data obtained are presented in Table II. It was thus found that, although the enzyme activity was not necessarily high, it was shown to double during the initial 3 hour periods of germination.

DISCUSSION

Prior to a discussion of the enzyme activities in cell-free extract of the fungal spores, it should be pointed out that the activities of the various enzymes studied are probably in some cases impaired in the preparation of the spore extract. There is ample evidence that the method of disintegration of cells affects enzyme activities according to the stability of the enzyme concerned. Indeed, in the present experiments also, the activities of pyrophosphatase, apyrase and polyphosphate-kinase of the spore extract were found to be very labile. When the sonicate of spores stood over night, even in the cold, these enzymes lost their activities almost completely.

In bacterial spores there are enzymes which become active when the spores are "activated", for example, by heat treatment. These enzymes are defined as "dormant enzyme" (*8*). Among several enzymes studied with these fungal spores, no enzymes which are inactive in dormant spores and activated by germination were found. All of them were shown to exhibit more or less activity even in the dormant state. Quantitatively, however, the rate of increase in activity seems to vary from one enzyme to another. Generally speaking, the activities of the enzymes involved in polyphosphate metabolism, which is considered to be especially important in the initiation of germination as reported earlier, increased considerably in the initial phase of germination. By contrast, the activities of phophomonoesterase and apyrase were found to be practically unchanged throughout the course of germination.

It has already been reported that the spores of a strain of *Aspergillus niger* showed only minor activities of pyro- and meta-phosphatase (*9*). Contrary to these observations, the strain of this fungus used in our laboratory exhibited definite pyrophosphatase and polyphosphatase activities. The fact that no pyrophosphate was detected in the acid-soluble fraction of spores during the course of germination (*1*, *2*) may be related to this finding. As discussed in part by Levinson (*10*), the physiological significance of pyrophosphatase in fungal spores, as well as in bacterial spores, may be involved in the elimination of pyrophosphate, to force to the "right" the various biochemical reactions producing pyrophosphate.

In the present experiment the polyphosphate-kinase activity of the cell-free extract of germinating spores was shown to be rather weak. It was demonstrated by quantitative analysis that only a small percentage of the polyphosphate phosphorus was transferred to ADP. It should, however, be recalled that in intact spores the polyphosphate phosphorus stored in dormant spores furnished part of the ATP phosphorus very rapidly in the initial period of germination (*2*). Thus it appears that the enzyme activity of the cell-free extract was partly inactivated in the preparation of the extract. On the other hand, the weak polyphosphate-kinase activity of the spore extract may, alternatively, be attributed to the use of synthetic polyphosphate as the substrate in this experiment, although the identity of the natural and synthetic polyphosphates has not yet been established. This line of consideration brings the role of polyphosphate-depolymerase in germinating spores into clear focus. As clearly demonstrated, this enzyme activity increased markedly in an initial phase of germination. This suggests that the natural polyphosphate contained in dormant spores is highly polymerized and that this macromolecular polyphosphate is depolymerized to an adequate molecular weight substance, which in turn is utilized for the formation of ATP catalized by polyphosphate-kinase.

SUMMARY

Enzyme activities involved in phosphorus metabolism in germinating spores of *Aspergillus niger* were investigated. A cell-free extract of the spores showed hydrolyzing activities on pyrophosphate, β-glycerophosphate, ATP, and polyphosphate. The spore extract also can transfer phosphorus from polyphosphate to ADP, producing ATP. The activities of these enzymes, except phosphomonoesterase and apyrase increased markedly during the course of germination, especially in an early period.

The author wishes to thank to Prof. T. Yanagita for his valuable advice in this work.

REFERENCES

(*1*) Nishi, A., *Annual Report of the Institute of Food Microbiology, Chiba University*, **12**, 32 (1959)
(*2*) Nishi, A., *J. Bacteriol.*, in press
(*3*) Yanagita, T., and Yamagishi, S., *Appl. Microbiol.*, **6**, 375 (1958)
(*4*) Akamatsu, S., and Katsumata, I., *J. Biochem.*, **39**, 207 (1952)
(*5*) Fiske, C. H., and SubbaRow, Y., *J. Biol. Chem.*, **66**, 375 (1925)
(*6*) Kornberg, S. R., *Biochim. et Biophys. Acta*, **26**, 294 (1957)
(*7*) Ebel, J. P., *Bull. Soc. Chim. Biol.*, **34**, 321 (1952)
(*8*) Halvorson, H., and Church, B., *Bacteriol. Rev.*, **21**, 112 (1957)

(*9*) Krishnan, P. S., and Bajaj, V., *Arch. Biochem. Biophys.*, **47**, 39 (1953)

(*10*) Levinson, H. S., "Spores," American Institute of Biological Sciences, Washington, D. C., p., 120 (1957)

The Journal of Biochemistry, Vol. 48. No. 5, 1960

CHOLINE ACETYLASE OF HUMAN PLACENTA

By JUNZO KATO

(From the Department of Obstetrics and Gynecology, Faculty of Medicine, University of Tokyo, Tokyo)

(Received for publication, May 23, 1960)

Comline (*1*) has demonstrated that the human placenta contains a considerable amount of choline acetylase. The presence of choline acetylase has been considered to be correlated with so-called "excitable tissues", *e. g.*, nervous tissues, electric organs and some kind of muscles (*2*). Therefore, the discovery of the enzyme in the placenta raises a new question in the physiological role of ACh* and its related enzymes.

In the present communication, an attempt was made to purify choline acetylase from the human placenta at full term. Using the purified preparation some properties of the enzyme were studied.

The method for preparation is shown in Scheme I. The enzyme was purified 9.9 fold; its activity was 0.305 μmoles ACh per mg. protein per hour, measured by the method in Scheme I. The placental enzyme was found to be more labile than the brain enzyme prepared by the method of Kumagai and Ebashi (*4*) and was stabilized by cysteine.

SCHEME 1

Preparation of Purified Choline Acetylase of the Human Placental Tissue[2]

		Purification	Recovery
	Fresh human placental tissue was washed several times with ice-cold Ringer solution, added to equal volume of 0.03 M potassium phosphate buffer solution (pH 6.8) and homogenized for three minutes in a siliconized Warning blendor.		
	↓		
Residue discarded ←	Centrifuged for 20 min. at 9,000×g	(1)	(100)
	↓		
Residue discarded ←	Supernatant[1] acidified with acetic acid to pH 4.5 and chilled for 1 hr. at 0°. Centrifuged.		

---

* The following abbreviations are used: ACh for acetylcholine, CoA for coenzyme A, ATP for adenosine triphosphate, EDTA for ethylenediaminetetraacetic acid and Tris for tris-(hydroxymethyl) aminomethane.

	Supernatant neutralized with potassium bicarbonate to pH 6.8	2.2	96
Residue discarded	Ammonium sulfate added to final concentration of 1.14 *M*. Centrifuged.		
Supernatant discarded	Ammonium sulfate added to final concentration of 1.89 *M*. Centrifuged.		
	Residue washed three times with ammonium sulfate solution[1] of 1.67 *M*; this procedure repeated twice. Centrifuged.		
	Residue dissolved in a small amount of 0.03 *M* potassium phosphate buffer[1] (pH 6.8) and dialysed against 0.03 *M* potassium phosphate buffer[1] (pH 6.8). Then adjusted to pH 6.20 with acetic acid.	7.1	57
Supernatant discarded	Calcium phosphate gel added (3.3 mg./protein mg.). Centrifuged.		
Extract discarded	Residue extracted with 0.05 *M* potassium phosphate buffer (pH 7.5). Centrifuged.		
Residue discarded	Residue extracted with 0.2*M* potassium phosphate buffer (pH 8.2). Centrifuged.	8.6	38
Supernatant discarded	Ammonium sulphate added to final concentration of 1.89 *M*. Centrifuged.		
	Residue dissolved in a small amount of 0.03 *M* potassium phosphate buffer (pH 6.8) and dialysed against 0.03 *M* potassium phosphate buffer (pH 6.8).	9.9	24

1) Cysteine was added (final concentration, 1 m*M*)

2) All the procedures were carried out at 4° or lower. The enzyme activity was measured as follows. Reaction mixture contained in μmoles per ml.: potassium acetate 5, choline chloride 5; eserine sulfate 1, cysteine 15, potassium ATP 3, potassium phosphate buffer (pH 6.8) 30, CoA 10 units and crude aceto-CoA-kinase (acetone dried powder derived from dog kidney) 15 mg. Incubated for 60 minutes at 38°. The amount of ACh formed in the testing mixture was assayed by the *rectus abdominis* muscle of the frog (*3*).

This lability of the placental enzyme was reflected in the remarkable stimulating effect of cysteine. As shown in Fig. 1, cysteine increased the activity of the placental enzyme five times, but that of the brain enzyme only about two times.

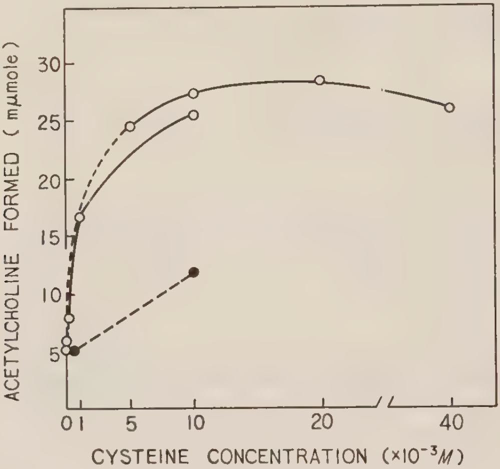


Fig. 1. Activating effect of cysteine on the choline acetylase activity.

—○— placenta choline acetylase
—●— brain choline acetylase

Reaction mixture contained the following components in μmoles per ml.: choline chloride 5; acetyl CoA 0.033; potassium phosphate buffer 46; the enzyme 390μg. of protein in the case of placenta choline acetylase and 530 μg. in the case of brain choline acetylase. The final pH was 7.1. The final mixture of 0.5 ml. was incubated at 15 minutes at 31°. The ACh formed was assayed by the biological method (*3*).

Reisberg (*5*) has shown the activating effect of EDTA on the choline acetylase of squid head ganglia. The placental enzyme was activated about 60 per cent by EDTA in the absence of cysteine.

In the presence of cysteine, *i.e.*, in the activated state of the enzyme, EDTA exerted much less but still definite stimulating effect on this enzyme.

Magnesium and calcium ions exerted no significant effect on the activity of the enzyme. Fairly high concentration of EDTA, *viz.*, 10 mM in sodium form, being only accelerating on this enzyme, it is obvious that even a trace of bivalent cations is not necessary for the function of this enzyme.

The optimum pH for this enzyme was 7.4–7.5 in the presence of cysteine (Fig. 2) and 7.7 in the absence of cysteine. It is worth of noting that Tris-buffer depressed the enzyme activity remarkably (Fig. 2). This contrasts strikingly with the results of Reisberg (*5*), who observed a remarkable stimulatory effect of Tris on this enzyme. The reason for this discrepancy remains to be solved.

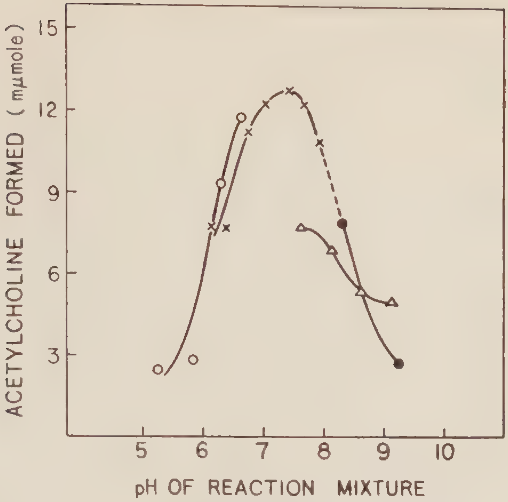


Fig. 2. Effect of pH on the activity of choline acetylase of the placenta in the presence of 10 mM cysteine.

—○— 0.030 M–0.033 M citrate phosphate buffer.
—×— 0.046 M potassium phosphate buffer.
—△— 0.04 M Tris buffer.
—●— 0.04 M glycine buffer.

Other details as in the legend for Fig. 1. except that half amounts of enzyme was used.

The author wishes to express his thanks to Prof. H. Kumagai (Dept. of Pharmacology) and Prof. T. Kobayashi (Dept. of Obstetrics and Gynecology) for their encouragement and to Prof. S. Ebashi (Dept. of Pharmacology) for his advice.

REFERENCES

(1) Comline, R. S., *J. Physiol.*, **105**, 6 (1946)
(2) Nachmansohn, D., and Wilson, I. B., *Advances in Enzymol.*, **12**, 159 (1951)
(3) Chang, H. C., and Gaddum, J. H., *J. Physiol.*, **79**, 255 (1933)
(4) Kumagai, H., and Ebashi, S., *Nature*, **173**, 871 (1954)
(5) Reisberg, R. Berman, *Biochem. et Biophys. Acta*, **14**, 442 (1954)

The Journal of Biochemistry, Vol. 48, No. 5, 1960

REDUCTION OF SULFITE TO SULFIDE IN MUNG BEAN LEAF

It has been suggested in some informations that sulfide may be an intermediate in a biosynthetic pathway of sulfur-containing amino acids from sulfate. Schlossman and Lynen reported that cysteine was synthesized by a condensation of serine and sulfide (*1*), and Hilz and Kittler demonstrated that sulfate was reduced to sulfide in the presence of adenosine triphosphate in yeast extract (*2*). Since Wilson and Bandurski showed a reduction of sulfate to sulfite in yeast extrace (*3*) and it was suggested in our experiments that sulfite might be an intermediate in a biosynthitic pathway of sulfur-containing amino acids from sulfate (*4*), so it is expected that sulfite may be reduced to sulfide in organisms. Actually Hilz and his coworkers have reported the separation of enzyme system capable of reducing sulfate to sulfite from the extract which is able to reduce sulfate to sulfide (*5*). This paper shows the formation of sulfide from sulfite in excised leaves of mung bean.

It was demonstrated in the previous paper that sulfite-S^{35} was incorporated into cysteine and some organic compounds in excised leaves of mung bean (*4*). As shown in Tabe, it was found in a recent work that a simultaneous infiltration of non-labeled sulfide with S^{35}-labeled sulfite into excised

	Radioactivity / g. of leaves			
	Exp. I		Exp. II	
Filtrated Substance	$S^{35}O_3^{--}$	$S^{35}O_3^{--}+S^{--}$	$S^{35}O_3^{--}$	$S^{35}O_3^{--}+S^{--}$
	c.p.m.	c.p.m.	c.p.m.	c.p.m.
Total absobed S^{35}	2.4×10^5	4.6×10^4	2.4×10^5	4.9×10^4
Sulfite and sulfate-S^{35}	2.1×10^5	4.6×10^4	2.1×10^5	4.9×10^4
Cystine-S^{35}	7600	24	1510	18
Sulfide-S^{35}	22	735	68	5400

The excised leaves were incubated in 20 ml. of a solution containing S^{35}-sodium sulfite at a concentration of 1 μc per ml., $5\times10^{-3}M$ non-labeled sodium sulfite and 0.01% of ethylenediaminetetraacetate for 3 hours under anaerobic condition. In case of the simultaneous infiltration of non-labeled sulfide, the incubated solution contained potassium hydrosulfide at $3\times10^{-2}M$. Total S^{35}, sulfite and sulfate-S^{35}, and cystine-S^{35} were determined as described in the previous paper (*4*), after treated with iodine solution. (Sulfite and sulfate-S^{35} might be almost sulfite-S^{35} before treating with iodine solution and could be contaminated with sulfide-S^{35} in this assay.)

leaves resulted in an inhibition of the incorporation of sulfite-S^{35} into cysteine and other organic compounds as well as an inhibition of the absorption of sulfite. The former inhibition may be due to either a poisonous action of sulfide or a preferential utilization of sulfide-sulfur as the source of sulfur-containing organic compounds.

The table also shows a reduction of sulfite to sulfide in this tissue. Eight g. of excised leaves were incubated in a solution containing S^{35}-labeled sulfite or both S^{35}-labeled sulfite and non-labeled sulfide for 3 hours under anaerobic condition, as described in the previous papers (*4*, *6*). The incubated leaves were homogenized with 4 ml. of 1 *M* NaOH and sulfide was separated by acidifying the homogenate, trapping volatile substances in an alkaline solution and repeating precipitation of $BaSO_3$. And then sulfide-sulfur was converted to methylene blue-sulfur, of which the radioactivity was measured after absorbing on charcoal (*7*). As seen in Table, some amount of sulfite-S^{35} was incorporated into sulfide only when non-labeled sulfide was infiltrated into excised leaves together with S^{35}-labeled sulfite. Since the previous paper has demonstrated that the metabolism of sulfate-S^{35} formed from sulfite-S^{35} by oxidation is neglegiable in this condition (*4*) and the amont of sulfide-S^{35} in Experiment II was 11 per cent of total absorbed isotope, labeled sulfide should be derived from S^{35}-labeled sulfite without oxidation to sulfate. Thus, the result suggests an operation of sulfite reductase system in this tissue.

REFERENCES

(*1*) Schlossman, K., and Lynen, F., *Biochem. Z.*, **328**, 591 (1957)
(*2*) Hilz, H., and Kittler, M., *Biochim. et Biophys. Acta*, **30**, 650 (1958)
(*3*) Wilson, L. G., and Bandurski, R. S., *J. Am. Chem. Soc.*, **80**, 5576 (1958)
(*4*) Asahi, T., and Minamikawa, T., *J. Biochem.*, **48**, 548 (1960)
(*5*) Hilz, H., Kittler, M., and Knape, G., *Biochem. Z.*, **332**, 151 (1959)
(*6*) Asahi, T., and Harada, N., *Bull. Agr. Chem. Soc. Japan*, **21**, 243 (1957)
(*7*) Koyama, J., and Nomura, M., "Tracer Technique," Nankodo, Tokyo, p. 100 (1955)

Laboratory of Biochemistry
Faculty of Agriculture
Nagoya University

TADASHI ASAHI

(Received for publication, May 16, 1960)

The Journal of Biochemistry, Vol. 48, No. 5, 1960

AMINO ACID INCORPORATION ENZYME IN POSTERIOR SILKGLAND

In the previous paper (*1*) we suggested the existence of an enzyme which was more concerned with the incorporation of amino acids into particulate fraction (RD) of silkgland than "pH 5 enzyme" of Hoagland *et al* (*2*). Recently Grossi and Moldave (*3*) demonstrated that the supernatant fraction of the isoelectric precipitation at pH 5 of rat liver catalyzed incorporation of C^{14}-amino acids from RNA-bound amino acid into ribonucleoprotein particles. In the present paper it is shown that there are at least two enzyme fractions involved in the incorporation of glycine-1-C^{14} into the particulate fraction of silkgland; one may be an enzyme which catalyzes the activation of amino acids, the other an enzyme which catalyzes the incorporation of activated amino acids into particulate fraction. Cellular fractions of the posterior silkgland of *Bombyx mori* were prepared by the method described previously (*1*). The crude enzyme fraction (E_2) was prepared by precipitation at pH 4.9 from a supernatant fraction obtained after 30 minutes centrifugation at $15{,}000 \times g$. The E_2 fraction was reprecipitated at pH 5.2 by addition of $0.2\,M$ acetic acid. This enzyme fraction was denoted as E_{21}. On the other hand, to the E_3 fraction, a supernatant obtained after centrifugation of E_2, cystalline ammonium sulfate was added to give 50 per cent saturation. After an hour the precipitate was centrifuged off and the supernatant fluid was brought to 60 per cent saturation. The precipitate was further purified by repeating ammonium sulfate precipitation at 60 per cent saturation. This fraction was denoted as N_6 and it had most of enzymic activity for the formation of glycine hydroxamate. The supernatant obtained at the first 60 per cent saturation was made to 80 per cent saturation and the resulting precipitate (N_8) was collected. The incorporation experiments were carried out as described previously (*1*).

The typical data are summarized in Table I and II. When RD (deoxycholate-treated particulate fraction) was incubated with E_2 or E_{21}, incorporation of glycine-1-C^{14} into the particulate protein was stimulated by E_2, but not by E_{21}. Such a loss of the activity of E_{21}, however, could be completely restored by addition of N_8 or E_3. E_{21} had no activity in catalyzing the formation of glycine hydroxamate and glycine-dependent exchange of PP^{32} with ATP. Although E_{21} contained approximately 18 per cent RNA, a soluble RNA fraction prepared by the method of Kirby from the supernatant obtained after centrifugation of the particulate fraction did not replace E_{21} as shown in Table II. E_{21} was inactivated by heating at 95° for 5 minutes at pH 8 in $0.05\,M$ Tris-HCl buffer, whereas

TABLE I

Effect of E_{21} Fraction on Incorporation of Glycine-C^{14} into Protein of Posterior Silkgland

Incorporation system	Radioactivity c.p.m. per mg. of protein			
	exp. 1	exp. 2	exp. 3	exp. 4
RD1)	13			
RD+$E_2$2)	155	113	151	
RD+E_{21}3)		10	14	13
RD+$E_3$4)	33		17	39
E_{21}+$N_8$5)		30		
RD+E_2+E_3	259		188	
RD+E_{21}+N_8		457	230	
RD+E_{21}+E_3			121	114
RD+E_{21}+$N_6$6)				71
RD+E_{21}+N_8		442		243

1) Deoxycholate-treated particulate fraction

2) Enzyme fraction precipitated at pH 4.9 from a supernatant fraction after removal of particulate fraction

3) Reprecipitated fraction of E_2 at pH 5.2

4) Supernatant obtained after centrifugation of E_2

5) Enzyme fraction which catalyzes mainly glycine-dependent exchange of PP^{32} with ATP

6) Enzyme fraction which catalyzes mainly formation of glycine hydroxamate

TABLE II

Effect of Soluble RNA on Incorporation of Glycine-C^{14} into Protein of Posterior Silkgland

Incorporation system	Radioactivity c.p.m. per mg. of protein	
	exp. 1	exp. 2
RD+E_{21}	86	13
RD+N_8	20	
RD+N_8+E_{21}	175	243
RD+N_8+S-RNA1)	12	
RD+E_{21}+N_8+S-RNA	151	
RD+E_{21}+S-RNA	87	
RD+E_3		39
RD+E_3+E_{21}		114
RD+E_3+S-RNA		37
RD+N_6+S-RNA		10

1) Soluble RNA

the soluble RNA was stable under the same condition in accepting amino acid from activating enzyme, agreeing with the results of Allen *et al.* (*4*). From these data, E_{21} may be a second enzyme which catalyze the incorporation of activated amino acid into the particulate protein.

On the other hand, the N_8 fraction appears to be an amino acid activating enzyme. This fraction, however, had little activity in the formation of glycine hydroxamate, although it catalyzed the glycine-dependent exchange of PP^{32} with ATP. This discrepancy and also the nature of the N_8 fraction are now under investigation.

REFERENCES

(*1*) Suzuka, I., and Shimura, K., *J. Biochem.*, **47**, 551, 555 (1960)
(*2*) Hoagland, M. B., Keller, E. B., and Zamecnik, P. C., *J. Biol. Chem.*, **218**, 345 (1956)
(*3*) Grossi, L. G., and Moldave, K., *Biochim. et Biophys. Acta*, **35**, 275 (1959)
(*4*) Allen, E. H., Glassman, E., Cordes, E., and Schweet, R. S., *J. Biol. Chem.*, **235**, 1068 (1960)

Laboratory of Biochemistry
Department of Agricultural Chemistry
Tohoku University, Sendai

IWAO SUZUKA
SHIGEAKI TANAKA
KENSUKE SHIMURA

(Received for publication, September 12, 1960)

The Journal of Biochemistry, Vol. 48, No. 5, 1960

EFFECT OF CHLORAMPHENICOL AND AMINO ACIDS ON STREPTOLYSIN FORMATION BY STREPTOCOCCAL GHOSTS

In a previous paper (*1*), ghosts of *Streptococcus pyogenes* S8 were shown to be able to produce streptolysin (streptolysin S′).

In the present paper, effects of chloramphenicol and amino acids on the toxin formation are described.

Table I shows that chloramphenicol inhibited streptolysin formation by ghosts.

TABLE I

Inhibition of Streptolysin Formation by Chloramphenicol

Concentration of Chloramphenicol	H. U.
0 (*M*)	390
1×10^{-6}	256
1×10^{-5}	185
1×10^{-4}	99
1×10^{-3}	0

The standard incubation medium contained 2 μmoles of $MgSO_3$, 30 μmoles of KH_2PO_4, 5 μmoles of maltose, 0.2 mmole of sodium succinate, 20 μmoles of thioglycolate and 100 μg. of the active fraction (*3*) (the oligonucleotide fraction obtained by ECTEOLA-cellulose column chromatography of pancreatic ribonuclease core of yeast ribonucleic acid) in a volume of 1 ml., pH being adjusted to 7.0. Ghosts prepared as described in the preceding paper (*1*) were suspended in the medium and incubated at 37° for 60 minutes in the presence of chloramphenicol of the indicated concentration. Reaction was stopped by chilling, ghosts were separated by centrifugation and hemolytic activity in the supernatant was determined with the use of a freshly prepared 3% rabbit erythrocytes suspension. The hemolytic unit (H. U.) is an amount of the hemolysin which can lyse half the erythrocytes contained in 1 ml. of phosphate buffered saline (pH 7.0) in 2 hours at 37°.

β-DL-phenylserine which is considered to be an analogue of phenylalanine was a strong inhibitor for the toxin formation and the inhibition was completely reversed by L-phenylalanine of the same concentration (Table II and III).

TABLE II

Inhibition of Streptolysin Formation by β-DL-phenylserine

concentration	H. U.
0 (*M*)	530
1×10^{-4}	21
5×10^{-4}	25
1×10^{-3}	29
3×10^{-3}	16

TABLE III

Reversal of β-DL-phenylserine Inhibition of Streptolysin Formation by L-phenylalanine

Concentration of phenylserine was 1×10^{-3} *M*

Concentration of phenylalanine added	H. U.
0 (*M*)	29
1×10^{-6}	144
1×10^{-5}	305
1×10^{-4}	350
1×10^{-3}	530
without phenylalanine and phenylserine	530

The effects of antibiotics and aminoacid analogues on the production of streptolysin by intact streptococcal cells were reported by Younathan and Barkulis (*2*). Our results obtained with a subcellular system were similar to their results and more direct insight into the mechanism of the toxin formation may be gained by our results.

In the course of these studies, we found that the addition of a mixture of amino acids increased the production of streptolysin by ghosts as well as by intact cells. This effect was not observed by Younathan and Barkulis.

The results presented in Table IV were obtained with the use of an acid hydrolyzed casein (Bacto Casamino acid) as an amino acid mixture. The possibility that the effect might be due to a minor component (or components) other than amino acids in the preparation was excluded because a mixture of purified 17 amino acids had an effect similar to Casamino acid.

Table IV

Effect of An Amino Acid Mixture on Streptolysin Formation

Concentration of amino acid mixture	H. U.
0 mg./ml.	206
0.5	284
1	284
2	430
5	512
10	2048
20	2048

These results seem to indicate that the streptolysin formation is connected directly or indirectly with protein or polypeptide formation.

The expense of this study was defrayed in part by a grant from the Ministry of Education.

REFERENCES

(1) S. Sugai, and F. Egami, *Proc. Japan Acad.*, **36**, 141 (1960)
(2) E. S. Younathan, and S. S. Barkulis, *J. Bacteriol.*, **74**, 151 (1957)
(3) T. Tanaka, *J. Biochem.*, **45**, 109 (1958)

Department of Biophysics and Biochemistry,
Faculty of Science, University of Tokyo,
Tokyo

SADAKO SUGAI
FUJIO EGAMI

(Received for publication, September 20, 1960)